Rhesus macaque KIR recognition of MHC class I molecules: Ligand identification and modulation of interaction by SIV peptides

Rhesus macaque KIR recognition of MHC class I molecules: Ligand identification and modulation of interaction by SIV peptides

A dissertation presented

by

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to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Virology

Harvard University

Cambridge, Massachusetts

May 2014

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Rhesus macaque KIR recognition of MHC class I molecules: Ligand identification and modulation of interaction by SIV peptides

Abstract

Natural killer (NK) cells can kill virus-infected cells without prior antigenic exposure, and are therefore important for controlling viral replication prior to the onset of adaptive immune responses. Primate NK cells express activating and inhibitory killer-cell immunoglobulin-like receptors (KIRs) that bind to specific major histocompatibility complex (MHC) class I molecules. The importance of KIR interactions with MHC class I in human immunodeficiency virus (HIV) pathogenesis is demonstrated by the association of select KIR and MHC class I genotypes with delayed progression to acquired immunodeficiency syndrome (AIDS).

Simian immunodeficiency virus (SIV) infection of rhesus macaques is the primary model for AIDS, but few KIR ligands have been defined in this species. We identified Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 as ligands for Mamu-KIR3DL01, one of the most polymorphic and commonly expressed KIRs in the rhesus macaque. We defined the MHC class I residues that are essential for this interaction, revealing an overlapping but distinct pattern of binding when compared to the recognition of ligands by human KIR3DL1, despite the lack of an orthologous relationship between these KIRs.

Peptide presented by MHC class I ligands can affect binding to inhibitory KIRs. This effect is of particular interest during viral infection, which significantly alters the repertoire of peptides presented by MHC class I on infected cells. Using a cell line that presents distinct Mamu-A1*002-peptide complexes, we demonstrated that Mamu-KIR3DL05^{+} NK cells were inhibited by Mamu-A1*002 presenting some SIV peptides, but not others. Approximately one

third of the SIV peptides previously shown to bind to Mamu-A1*002 had an inhibitory effect upon Mamu-KIR3DL05^{+} NK cell activity. Among the inhibitory peptides were the three SIV CD8^{+} T cell epitopes that bind to Mamu-A1*002 with the highest affinity. Analysis of peptide variants revealed that residues 7 and 8 are most important for modulating interaction with Mamu-KIR3DL05. Peptide mixture experiments showed that the effect of an inhibitory peptide was dominant over a non-inhibitory peptide to prevent Mamu-KIR3DL05^{+} NK cell activation. These experiments suggest that inhibitory viral epitopes may prevent killing of infected cells by Mamu-KIR3DL05^{+} NK cells as a mechanism of immune evasion.

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Acknowledgements

First and foremost, I thank David Evans for giving me the opportunity to work on this project and for providing guidance and support during my graduate studies. I also thank past and present members of the Evans lab, especially Arnaud Colantonio, Natasha Guha, and Bill Neidermyer with whom I worked on the KIR project, and Ruth Serra-Moreno and Michael Alpert for many great conversations scientific and otherwise.

I would also like to thank the members of my Disseration Advisory Committee, Drs. Dana Gabuzda, Marcus Altfeld, and Amitinder Kaur, and my thesis examiners, Drs. Todd Allen, Sylvie Le Gall, and Kenneth Williams. I am also grateful to the NEPRC faculty and staff past and present who created a wonderful collaborative research environment while it lasted and were always willing to offer advice. I also thank my undergraduate mentors Jay Nadeau and Greg Matlashewski for giving me a start in science when I had no idea what I was doing.

I am grateful to students in the Virology program who have made my graduate school experience so much richer, from helpful criticism at Data Club to the occasional epic dance party during recruitment or retreat. Thank you especially to my classmates who have been my carpooling buddies, travel companions, roommates, and Clue costume compatriots.

Most of all, I am grateful to my family for their unending support. I thank my husband Dan Dwyer for being a constant during the ups and downs of research - I am so glad that we're a team. Finally, I must thank my parents, who have encouraged me as I moved across the country and then out of it to pursue my studies, even though they would have much rather kept me close to home. They also get bonus points for reading my scientific papers.

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Chapter 1

Natural Killer Cells and Immunodeficiency Virus Infection

1.1 - The Immunodeficiency Viruses

Human immunodeficiency virus (HIV) infection, if untreated, leads to acquired immunodeficiency syndrome (AIDS) and death (1). Approximately 35 million people worldwide are currently HIV-positive (2). HIV is a lentivirus of the retrovirus family. Other lentiviruses include simian immunodeficiency virus (SIV) (3) and feline immunodeficiency virus (FIV) (4). There are two types of HIV, HIV-1, which is closely related to SIV from chimpanzees (5), and HIV-2, which is closely related to SIV from sooty mangabeys (6). HIV-1 can be divided into three groups: M (main), O (outlier) and N (non-M or O). Of these groups, M is responsible for the worldwide epidemic of HIV infection. HIV-1 group M can be further subdivided into at least nine phylogenetically distinct clades: A, B, C, D, F, G, H, J, and K (7). These clades are geographically divided, such that clade B infection is most prevalent in North America and Europe, whereas clade C infection is the most common worldwide due to its prevalence in southern Africa (8).

Immunodeficiency Virus Replication

Both HIV and SIV genomes are flanked by long terminal repeats (LTRs) and contain three main open reading frames (ORFs): *Gag*, *Pol*, and *Env* (Figure 1.1) (9). The Gag-Pol polyprotein can be cleaved into seven proteins: matrix, capsid, nucleocapsid, p6, protease, reverse-transcriptase, and integrase (10). *Env* is translated into the gp160 envelope protein that is cleaved to gp120 surface and gp41 transmembrane proteins. HIV and SIV also share five smaller ORFs: *Vif*, *Vpr*, *Tat*, *Rev*, and *Nef* (9). However, HIV-1 and SIV differ with regards to two gene products: HIV-1 encodes Vpu, whereas SIV does not, and SIV encodes Vpx, whereas HIV-1 does not. Vif, Nef, Vpu, and Vpx are accessory proteins that do not play direct roles in the viral

life cycle. Vif causes ubiquitination and degradation of the host restriction factor APOBEC3G that causes G to A hypermutation in the viral genome (11, 12). Nef downregulates several cell surface proteins to optimize the infected cell activation for viral production and to evade $CD8^+$ T cell responses by MHC class I downregulation (13). In addition, Nef from SIVmac (14) and HIV-1 Vpu (15) downregulate tetherin, a host restriction factor that decreases viral release. Finally, SIV Vpx induces proteasomal degradation of SAMHD1, which is expressed in macrophages and dendritic cells and depletes the cellular pool of dNTPs (16, 17). The role of the remaining viral proteins involved in replication will be described below.

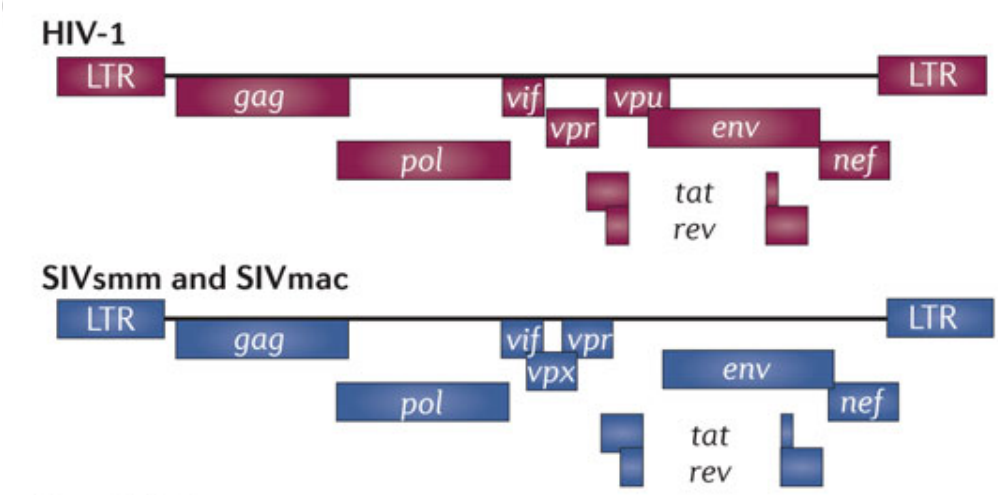


Figure 1.1. Genome organization of HIV and SIV. Reproduced with permission from (9). © 2012 Macmillan Publishers Ltd.

Infection with HIV or SIV begins with binding of gp120 to its CD4 receptor on the cell surface (10). This binding event causes a conformational change that allows gp120 binding to the coreceptor, usually CCR5, and subsequent fusion of viral and cellular membranes. Fusion releases the viral capsid into the host cytoplasm where partial viral uncoating occurs and reverse transcriptase produces dsDNA from the viral RNA template (10). This dsDNA genome is transported into the nucleus as part of the pre-integration complex including viral DNA, Vpr,

matrix, and integrase (18, 19). Integrase then inserts the viral genome into the host chromosome. Transcription of viral genes occurs via host machinery, and splicing leads to production of Rev, Tat, and Nef. Tat enhances viral transcription elongation (20), while Rev promotes nuclear export of unspliced mRNAs to allow translation of other viral proteins and incorporation of the full viral genome into new particles (21, 22). The Gag precursor protein binds to viral genomic RNA via the nucleocapsid region and accumulation of Gag and Gag-Pol at the cell membrane where Env is also localized leads to viral budding. Cleavage of Gag by protease after budding yields mature viral particles (10).

1.2 - The Immunology of Immunodeficiency Virus Infection

No cure or vaccine is yet available for HIV infection (23). Research into the pathogenesis of HIV, immune responses to the virus, and potential vaccine strategies is therefore of the highest priority. Rhesus macaque infection with simian immunodeficiency virus (SIV) also results in AIDS (3, 24) and is the primary animal model for AIDS research, particularly with respect to vaccine development. A highly-pathogenic clone of SIV, SIVmac239, is often used as the challenge strain in vaccine candidate studies (24).

Clinical Course of HIV Infection

The primary cell type infected by HIV and SIV is the $CD4^+$ T cell, though macrophages (25-28), dendritic cells (DCs) (29, 30), astrocytes (31-33), and a subset of CD4-expressing NK cells (34) are also infected. In the weeks following infection, HIV viral load increases to a peak of about 10^7 viral copies per ml of blood and $CD4^+$ T cell count decreases to about 500 cells per μL, compared to 1000 cells/μL in a healthy individual (35). The immune response cascade in

HIV infection begins with production of type I interferon (IFN) by DCs, followed by IL-15 production that promotes the subsequent proliferation of natural killer (NK) cells (Figure 1.2) (36). The role of NK cells during acute infection is two-fold. Because NK cells can kill virus-infected cells without prior antigen exposure and release chemokines that can block HIV coreceptor binding, they may limit viral replication prior to the onset of adaptive immune responses (37, 38). Furthermore, NK cells produce cytokines, such as IFN-γ, that can contribute to the development of a $CD8^+$ T cell response even in the absence of $CD4^+$ T cells as demonstrated in murine models (39). The peak of NK cells and $CD8^+$ T cell responses correlates with a decrease in viral titer and rebound in $CD4^+$ T cell count that marks the onset of clinical latency (40). The importance of such cellular responses is highlighted by the association of $CD8^+$ T cell response with control of viral replication and delay of disease (41-43) and the increase in SIV replication in rhesus macaques following CD8-depletion, which removes both $CD8^+$ T cells and NK cells (44).

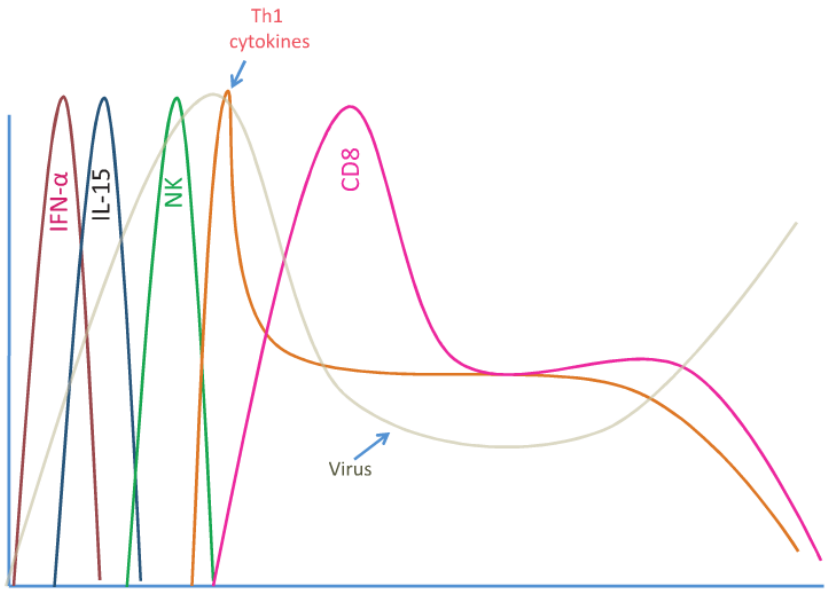


Figure 1.2. Cascade of immune responses in HIV infection. Described in text. Reprinted with permission from (36). © 2008 Blackwell Publishing Ltd.

Clinical latency can last for 10-15 years in HIV infection (45, 46), and 1-2 years following SIV infection of rhesus macaques (24, 47). During this time viral titer in the blood may be two to three logs lower than in the acute phase, but viral replication persists and causes $CD4^+$ T cell turnover (48). The set-point at which the viral titer plateaus in the latent phase of infection is strongly associated with disease progression (49). Less than 1% infected individuals have extremely low viral set points, some below the level of detection by polymerase chain reaction (PCR) assay, and many of these patients can live without antiviral therapy and without progression to AIDS for decades (50-52). However, without treatment most patients eventually progress to AIDS, when $CD4^+$ T cell counts decrease again and viral loads increase. $CD8^+$ T cell responses also fail during this late chronic phase. These immunopathogenic effects of HIV infection lead to numerous infections in late-stage disease. AIDS is marked by opportunistic infections such as *Pneumocystis jiroveci* and candidiasis (53). There are also several AIDS-associated cancers, including Kaposi's sarcoma, caused by Kaposi's sarcoma-associated herpesvirus, and Burkitt's lymphoma, caused by EBV (54). Neurological symptoms, including dementia, and infections of the nervous system, such as cryptococcal meningitis, are also prevalent in chronic AIDS patients (55).

The clinical course of HIV infection has changed dramatically with the introduction of antiretroviral drugs, beginning with azidothymidine (AZT) (56). Today inhibitors of viral protease, reverse transcriptase, integrase, and entry can be combined in a multi-drug regimen that completely suppresses viral replication below the limit of detection (57). However, such treatment is not sufficient to eradicate the virus, as viral load quickly rebounds after medication is halted. This rebound arises from viral reservoirs in long-lived cells containing latent integrated provirus. The main population of cells harboring the latent virus is resting memory $CD4^+$ T cells

(57-59), which may live for years without being activated - an event that would cause subsequent viral gene expression and cell death. Yet this $CD4^+$ T cell population does not account for all virus that rebounds after cessation of treatment, suggesting the existence of other reservoirs (60). Monocytes, DCs, and hematopoietic stem cells may be among these other reservoirs (61). It had been calculated that due to the slow turnover of these reservoir cells, complete viral suppression by antiretroviral treatment for 73 years would be sufficient to eliminate all viral reservoirs (62). Yet recent studies suggest that the amount of replication-competent latent virus was underestimated in this calculation and full clearance would take even longer (63). Clearly drug treatment alone will not be sufficient to cure HIV, but there is some hope that drug treatment in combination with reactivation of viral reservoirs and promotion of immune responses may lead to a functional cure (64). Better characterization of the regulation of NK cell and $CD8^+$ T cell activity may inform strategies to promote killing of infected cells that would be useful in this context.

Immune Responses to Immunodeficiency Viruses

$CD8^+$ T cell responses. $CD8^+$ T cell responses are critical for controlling HIV and SIV viral replication and are associated with delayed progression to disease in untreated individuals (41-44). HIV-specific $CD8^+$ T cells recognize infected cells that present MHC class I-bound HIV epitopes and kill them by releasing perforin and granzyme (65, 66). $CD8^+$ T cells also secrete chemokines that may inhibit viral entry (67). Nef-mediated downregulation of major histocompatibility complex (MHC) class I (68) and viral sequence variation to escape TCR recognition are the main mechanisms by which the virus evades this response. $CD8^+$ T cell responses to certain Gag epitopes are associated with positive disease outcome (69, 70). MHC

class I molecules associated with delayed disease progression, including Mamu-A1*001, -B*008 and -B*017 in rhesus macaques (71-73) and HLA-B*27 and -B*57 in humans (74, 75), may present viral epitopes that elicit a particularly effective $CD8^+$ T cell response from which the virus cannot escape without great fitness cost (76-80). Despite the effectiveness of $CD8^+$ T cells in controlling acute viral infection, $CD8^+$ T cells can become exhausted during chronic HIV infection and upregulate when PD-1, correlating with disease progression (81). In contrast, non-progressors maintain strong $CD8^+$ T cell proliferative capacity despite long-term HIV infection (82). The $CD8^+$ T cell response therefore seems critical for initial viral control but may become less effective as proliferative capacity is lost in progressive HIV infection, during which high levels of viral replication can persist despite prevalent HIV-specific $CD8^+$ T cell responses (83-85).

$CD4^+$ T cell responses. The efficacy of HIV-specific $CD4^+$ T cell responses is complicated by the fact that these cells are also the targets of HIV infection. HIV-specific cells are more highly infected than other CD4+ T cells, and any antigen-induced proliferative response would create more targets for viral infection (86). Likewise, the association of $CD4^+$ T cell function with elite control (87) may simply be a result of decreased infection of these cells, a hypothesis supported by the finding that patients on antiretroviral therapy also have greater $CD4^+$ T cell function (88). Still, several $CD4^+$ T cell functions play a role in developing effective responses to HIV: helper T cells enhance $CD8^+$ T cell responses (89), T follicular helper cells promote B cell antibody production (90), and cytolytic $CD4^+$ T cells kill virus-infected cells (91).

Antibody responses. Although antibodies emerge in the weeks following HIV infection, neutralizing antibodies are not generally protective. Neutralizing antibodies are actually less common in HIV non-progressors than in viremic individuals (92), yet a small subset of

individuals produces broadly neutralizing antibodies during controlled infection (93). These antibodies are capable of neutralizing a wide variety of HIV strains, and as such may provide a protective effect not afforded by other antibodies. Antibodies targeting the CD4 binding site of gp120, variable loops of gp120, or a membrane-proximal region of gp41 are the most effective (94). Furthermore, non-neutralizing functions of antibodies may play a role in minimizing viral acquisition or replication, particularly in the case of antibody-dependent cell-mediated cytotoxicity (ADCC). Protection after vaccination with live-attenuated SIV is associated with ADCC activity (95). Envelope glycosylation, which prevents antibody recognition of much of the viral envelope, is a critical viral immune evasion strategy (96, 97). In addition, the high degree of antigenic variation makes it difficult to produce an antibody that can recognize all variants of the virus found within an individual, leading to quick emergence of resistance to mounted responses.

Natural killer cell responses. NK cells are primarily activated to kill virus-infected cells and tumor cells that have low expression of MHC class I molecules that act as ligands for NK cell inhibitory receptors. Because MHC class I is downregulated by Nef in HIV and SIV, infected cells are vulnerable to killing by NK cells. However, HIV selectively downregulates HLA-A and -B, but not HLA-C, which is largely responsible for inhibiting NK cell function in humans through interaction with inhibitory KIR2D molecules (98). This selective downregulation allows evasion of HLA-A- and -B-restricted $CD8^+$ T cell responses and maintenance of HLA-C expression for inhibition of NK cells. However, rhesus macaques do no express orthologues of either HLA-C or KIR2D, and although Nef selectively downregulates some Mamu-A and -B molecules (99), there is no evidence to suggest that this selective downregulation maintains NK cell inhibition. In addition to being activated by loss of MHC

class I-mediated inhibitory signals, NK cells may also be activated by the upregulation of activating receptor ligands on infected cells (100). Finally, NK cells can be activated to kill cells that bind antibody that in turn binds CD16 on the NK cell. This interaction is the functional mechanism behind the ADCC effect of non-neutralizing antibodies described above. The regulation of NK cells and their role in HIV and SIV infection will be discussed in greater detail in the following sections.

1.3 - Natural Killer Cells and Killer-cell Immunoglobulin-Like Receptors

NK cells are essential for controlling many viral infections (101-103). NK cells are regulated by activating and inhibitory receptors on the cell surface that allow them to recognize some virus-infected and tumor cells without previous exposure to a given antigen. The balance of signals from NK receptors binding ligands on a target cell determines whether an NK cell will become activated (104). Upon activation, an NK cell can kill target cells by releasing cytotoxic granules containing perforin and granzyme or through expression of Fas-L and TRAIL (105). NK cells may also produce cytokines, including interferon (IFN)-γ (106), and β-chemokines, including CC-chemokine ligand 3 (CCL3), CCL4, and CCL5 (37, 107, 108). NK cells are particularly important in early control of viral infection prior to development of antigen-specific adaptive immune responses (36).

NK Cell Subsets

Human NK cells are divided into different functional subsets based on expression of CD16 and CD56. $CD16^{+}CD56^{dim}$ cells are more cytotoxic and produce limited cytokine (109), whereas $CD16^{-}CD56^{bright}$ NK cells are thought to play a regulatory role (110). However, rhesus

macaque NK cells differ from those in humans, as most do not express CD56 (111). Instead, a rare population of $CD56^{+}$ NK cells in rhesus macaques correspond to the $CD56^{bright}$ population from humans, expressing lymph node homing markers CCR7 and CD62L and low levels of granzyme B and perforin (Figure 1.3). A rhesus macaque $CD16^{+}CD56^{-}$ NK cell subset corresponds to the $CD16^{+}CD56^{dim}$ human population, lacking expression of lymph node homing markers and maintaining relatively high expression of granzyme B, perforin, and β-chemokines (112-114). A third population of $CD16^{-}CD56^{-}$ NK cells appear to be an intermediate stage between the other two subsets, which is consistent with the hypothesis that rhesus macaque $CD56^{+}$ and human $CD56^{bright}$ NK cells are immature (115).

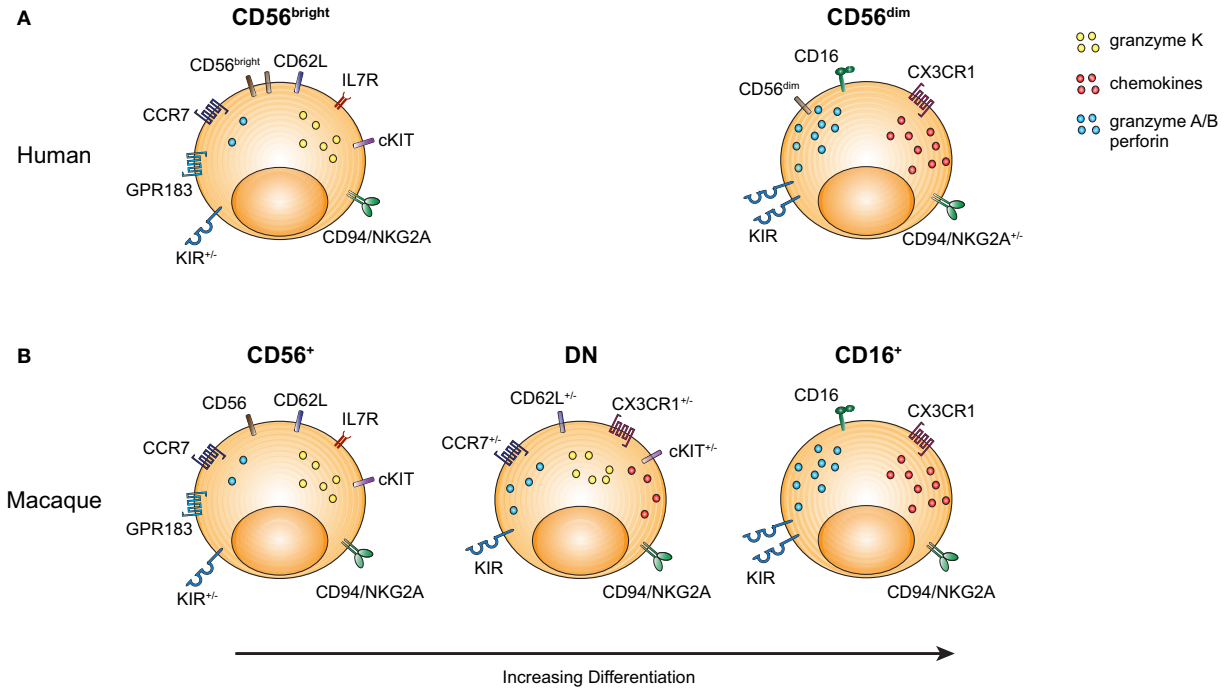


Figure 1.3. Human and rhesus macaque NK cell subsets. (**A**) Human NK cell subsets described by $CD56^{bright}$ or $CD56^{dim}$ expression have distinct functional capacities and expression of several cell surface markers. (**B**) Rhesus macaque NK cell subsets described as $CD56^{+}$ and double negative (DN) are less differentiated than mature $CD16^{+}$ NK cells. Each subset has distinct cell surface markers and functional capacity. Reproduced under Creative Commons Attribution License from (114).

Regulation of NK Cell Activity

In primates, NK cell receptors that recognize MHC class I ligands belong to two families: killer-cell immunoglobulin-like receptors (KIRs) and killer lectin-like receptors (KLRs). KLRs in primates include CD94/NKG2 heterodimers that recognize HLA-E in humans and are inhibitory, in the case of NKG2A, or activating, in the case of NKG2C (116-118). Another KLR, NKG2D, is a distinct type of activating receptor that recognizes stress-induced MHC class I-like molecules such as MICA/B and ULBP (119, 120). Expression of these NKG2D ligands can be induced by some viral infections, leading to NK cell activation. HIV evades this response through Vpu- and Nef-mediated downregulation of NKG2D ligands (121, 122). Unlike KLRs that recognize relatively conserved MHC class I and MHC class I-like molecules, KIRs specifically recognize highly polymorphic ligands HLA-A, -B, and -C in humans, and Mamu-A and -B ligands in rhesus macaques. Correspondingly, KIR are by far the most polymorphic of the NK cell receptors (123). In addition to being expressed on NK cells, KIRs are also expressed on a subset of $CD8^+$ T cells (124) and may therefore modulate both NK cell and cytolytic T cell responses. Notably, mice do not express KIRs but instead use Ly49, a KLR, as the primary polymorphic receptor for MHC class I ligands (125).

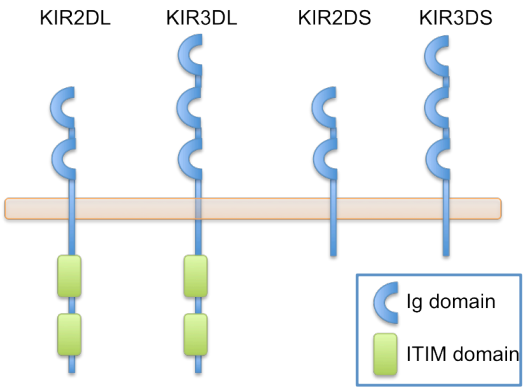


Figure 1.4. KIR structure. Graphic representation of inhibitory (L) and activating (S) KIRs with two (2D) or three (3D) extracellular Ig domains.

KIRs are type I transmembrane proteins that may have two (2D) or three (3D) extracellular immunoglobulin (Ig)-like domains (126, 127), and a short (S) or a long (L) cytoplasmic tail (128), as diagrammed in Figure 1.4. The Ig-like domains are D0, found in KIR3D molecules only, D1, and D2. Long cytoplasmic tails belong to inhibitory KIRs and contain two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). In contrast, short cytoplasmic tails belong to activating KIRs, which associate with an adaptor molecule that has an immunoreceptor tyrosine-based activating motif (ITAM) in its cytoplasmic tail (128). Human KIR3DS1 associates with the DAP12 adaptor through a trasmembrane lysine residue, but rhesus macaque activating KIRs lack this lysine and the adaptor for these molecules is unknown. Upon activating receptor engagement, ITAMs in the adaptor protein are phosphorylated by Src kinases, which leads to the recruitment of Syk or ZAP70 and the initiation of a phosphorylation cascade leading to cell activation (129, 130). In contrast, after inhibitory receptor binding, ITIMs recruit SHP-1 and SHP-2 phosphatases, dephosphorylating components of the ITAM cascade to prevent activation (131). Thus, a balance of the two signals determines NK cell activity.

Given the balance of signals regulating NK cell activity, NK cell activation can result from a loss of inhibition or an increase in activating signal. When interacting with a healthy autologous cell, NK cells are inhibited by binding of inhibitory receptors to self-MHC class I molecules (Figure 1.5A). To ensure self-tolerance, NK cells stochastically express KIR during development until there is sufficient expression of inhibitory receptors that recognize self-MHC class I molecules to prevent killing of autologous cells (132). NK cells that do not become sufficiently inhibited remain anergic. Once KIR expression has been established for a developing NK cell, expression is stably maintained by DNA methylation (133). This process creates a heterogeneous population of NK cells, some of which express multiple KIRs, that are inhibited

when interacting with healthy autologous cells. However, during tumor progression or viral infection, MHC class I expression on autologous cells may decrease (13, 134). These conditions can lead to NK cell activation by "missing self" or the lack of inhibition by autologous MHC class I molecules (Figure 1.5B). Alternatively, NK cells can be activated by excessive activating receptor engagement, even under conditions when inhibitory signals remain present (135, 136). Activating ligands that may be upregulated on virus-infected cells include NKG2D ligands MICA/B and ULBP, which are induced by stress (119, 120). Some NK cell activating receptors also detect specific viral gene products, such as NKp46, which binds to influenza hemagglutinin (HA) (137), and Ly49H in mice, which recognizes the murine cytomegalovirus protein m157 (138). NK cell activation by virus-infected cells results from a combination of these signals: loss of MHC class I recognition by inhibitory receptors, increased recognition of stress-induced ligands by activating receptors, and in some cases recognition of viral proteins by activating receptors.

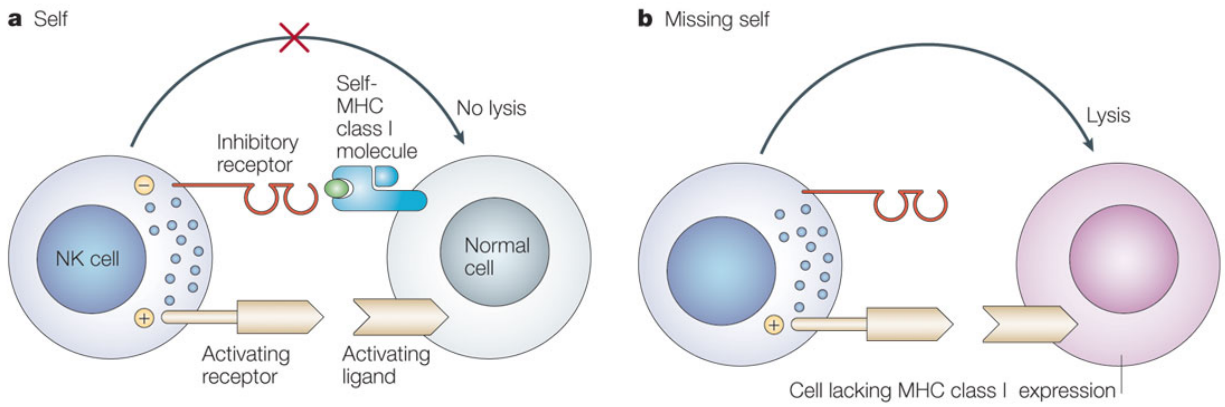


Figure 1.5. NK cell tolerance and missing self. Expression of self-MHC class I molecules prevents lysis by autologous NK cells. Decreased expression of these inhibitory receptor ligands can lead to "missing self," NK cell activation, and lysis. Reproduced with permission from (139). © 2005 Nature Publishing Group.

KIR Ligands in Humans and Rhesus Macaques

The ligands for many human KIRs have already been identified and can be defined in part by determinant residues in the MHC class I α1 domain. Human KIR2DL recognize HLA-C molecules that fall into two different categories. KIR2DL1 binds allotypes that have an asparagine at residue 77 and a lysine at residue 80, whereas KIR2DL2 and KIR2DL3 bind allotypes that have a serine at residue 77 and an asparagine at residue 80 (140). HLA-B molecules can also be divided into two groups based on residues in a similar region: Bw4 molecules have an NLR(I/T)ALR motif at residues residues 77-83 in the α1 domain and Bw6 molecules have an SLRNLRG motif at the same position. Human KIR3DL1 interacts with HLA-A and -B molecules that have a Bw4 motif (141, 142). In contrast, KIR recognition of HLA-B Bw6 molecules has not been observed. Unlike human KIR ligands, few rhesus macaque KIR ligands have been defined. Prior to the work included in this dissertation, Mamu-KIR3DL05 interaction with Mamu-A1*002 had been identified by binding and cellular assays (143, 144). Additional binding data indicates that Mamu-A1*001 and -A3*13 are also ligands for Mamu-KIR3DL05, and that Mamu-KIR3DLW03 binds Mamu-A1*001 and -A1*011 (143). Of note, though human KIR recognition of Bw6 molecules has not been demonstrated, these Mamu-KIR3D ligands include the Bw6 molecules Mamu-A1*002 and -A3*13. Although these interactions with inhibitory KIRs have been verified by binding assays, with the exception of a recent crystal structure of KIR2DS2 in complex with HLA-A*11 (145), such verification has been difficult for activating KIR because many exhibit low ligand binding or no detected binding (146). KIR3DS1, for example, is an inhibitory KIR with extracellular homology to KIR3DL1 and would therefore be expected to bind similar ligands. Indeed, in cellular assays KIR3DS1^{+}

NK cells are activated by target cells expressing Bw4 molecules that are ligands for KIR3DL1, but binding data to confirm this interaction has not been conclusive (147-149).

Determinants of KIR Recognition

Residues in the α1 and α2 domains of ligand MHC class I molecules are determinants of KIR recognition. Contact residues have been determined by crystals structures of human KIR-ligand complexes: KIR2DL2 with HLA-Cw3 (150); KIR2DL1 with HLA-Cw4 (151); KIR2DS2 with HLA-A*11 (145); and KIR3DL1 with HLA-B*57 (152). Consistent with other KIR structures, KIR3DL1 binds over the carboxy-terminus of the HLA-B*57 peptide-binding cleft, contacting both the α1 and α2 helices (Figure 1.6). As previously mentioned, recognition by KIR3DL1 is highly dependent on the Bw4 motif in the ligand α1 domain. The isoleucine at position 80 and the arginine at position 83 in this region are contacted by KIR3DL1 D1 and D2, respectively. Mutational analysis revealed that alanine substitution at either of these positions abrogated KIR3DL1 recognition, and that additional contact residues in the α2 helix are also essential for binding. KIR3DL1 also contacts the MHC class I-bound peptide. A leucine at position 166 in the D1 domain contacts the serine at position 8 of the HLA-B*57-bound peptide. Similarly, KIR2DL2 and KIR2DS2 also contact residue 8 in the HLA-bound peptides of their respective ligands (145, 150). Substitution at this peptide position can drastically alter the affinity of KIR binding, demonstrating the peptide selectivity of this interaction.

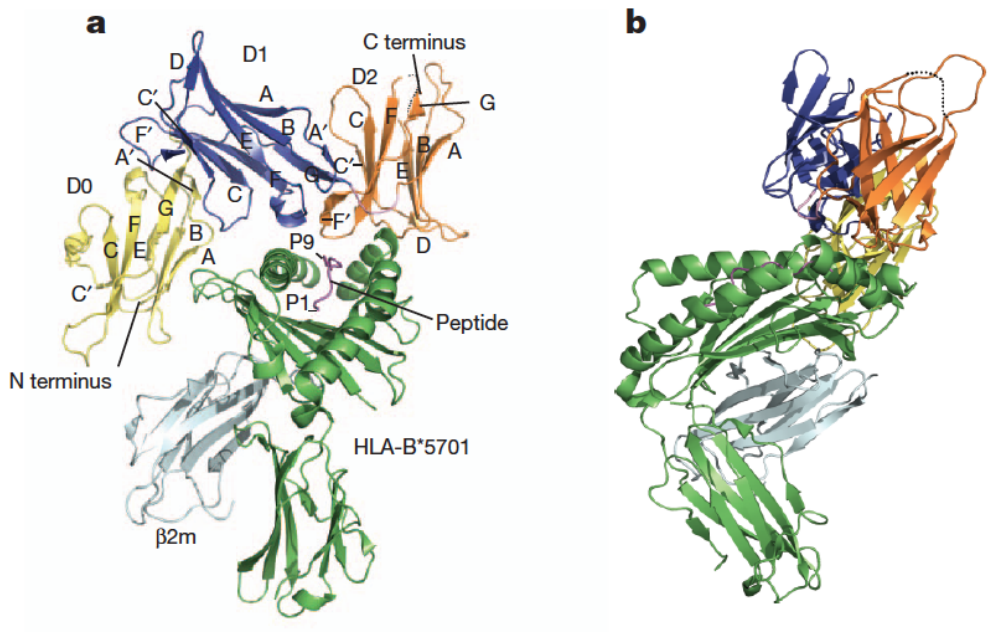


Figure 1.6. Crystal structure of human KIR3DL1 in complex with HLA-B*57. Orthogonal view of KIR3DL1 binding over the C-terminus of peptide presented by HLA-B*57. KIR3DL01 β-strands are labeled in (**a**). KIR3DL1 D0, D1, and D2 are colored yellow, blue, and orange, respectively. HLA-B*57, peptide, and β2m are colored green, purple, and grey, respectively. Reprinted with permission from (152). © 2011 Macmillan Publishers Ltd.

The peptides presented by MHC class I ligands affect recognition by inhibitory KIR. Human KIR3DL1 binds HLA-A*24 and -B*27 tetramers folded with some peptides but not others (142, 153, 154). Recognition by this KIR and affected by alterations at position 7 or 8 in HLA-B*27-bound peptides and the hierarchy of preferred peptides varies among different KIR3DL1 allotypes. Like KIR3DL1 binding, both KIR2DL1 and KIR2DS1 binding to HLA-Cw4 are altered by changes in residue 8, and to a lesser extent residue 7, of the presented peptide (155, 156), corresponding to the closer proximity of residue 8 sidechain to KIR2DL1 in the complex crystal structure (151). Furthermore, an antagonistic peptide presented by HLA-C can cause activation of NK cells by loss of KIR2DL2- and KIR2DL3-mediated inhibition (157). This peptide selectivity has implications during conditions of cell stress or viral infection, wherein the array of peptides presented on the cell surface may change dramatically (158, 159). In some

cases, presentation of viral peptides may lead to NK cell activation. HLA-Cw7 presenting peptides derived from glutamic acid decarboxylase or coxsackie virus, but not other self-peptides tested, could be killed by a KIR2DL2-expressing NK cell clone (160). These data highlight that KIR may be responsible not only for the recognition of "missing self" due to downregulated MHC class I, but also for recognition of "altered self" in the form of presentation of stress-induced or viral peptides.

1.4 - NK Cells in Immunodeficiency Virus Infection

While some NK cell subsets may become infected during HIV infection, NK cells are clearly more than just sites of viral replication during immunodeficiency virus infection. The vital role that NK cells may play in early infection is highlighted by the fact that the initial decrease in viral load is prior to the onset of adaptive immune responses and may therefore be an effect of NK cell control (36). Furthermore, an increase in SIV replication in infected animals was observed following depletion of $CD8^+$ cells, which includes both $CD8^+$ T cells and NK cells in rhesus macaques, thereby demonstrating the importance of one or both of these responses for SIV control (44). Transient depletion of $CD16^+$ cells in rhesus macaques during primary and chronic SIV infection attempted to specifically assess the importance of NK cells for SIV control and saw a trend toward higher viral load in primary infection, but no effect of depletion during chronic infection (161, 162). However, CD16 is not a true NK cell marker in rhesus macaques, and $CD16^-$ NK cell subsets are prevalent in the gut mucosa where much of SIV replication takes place (113). Moreover, CD16 is also expressed by monocytes, further complicating interpretation of these depletion results (163). Further studies with a more NK cell-specific depleting antibody will be necessary to determine the effect that these cells have on SIV control.

Changes in NK cells During Immunodeficiency Virus Infection

Many functional changes have been observed in NK cells during HIV and SIV infection. The frequencies of NK cell subsets are often altered. Broadly, decreasing lytic function of NK cells has been associated with AIDS and duration of HIV infection, and has been proposed as a prognostic indicator of progression to disease (164, 165). A decrease in $CD56^+$ NK cells, and a concomitant expansion of $CD56^{dim}CD16^+$ NK cells occurs during acute HIV infection, followed by depletion of the $CD16^+$ NK cells as HIV progresses and increased anergy in the remaining cells of this subset (164, 166-168). The impairment of NK cells in chronic HIV infection further extends to their ADCC function (169, 170) and cytokine secretion (171), whereas chemokine secretion is unaffected by infection (108). Although it has been suggested that HIV infection of NK cells contribute to these imbalances, they are equally cytotoxic as uninfected NK cells and there are therefore likely other mechanisms at play (172, 173). Notably, HIV controllers maintain highly-functional NK cells, which may contribute to sustained control of viral replication (174).

In addition to changes in the frequency of NK cell subsets, immunodeficiency virus infection can alter NK cell trafficking. In SIV infection, $\alpha4\beta7$ expression on NK cells increases and CCR7 expression decreases, increasing trafficking to the gut mucosa and decreasing lymph node homing, respectively (175). During SIV infection, $CD56^+$ NK cells become redistributed to the gut, whereas $CD16^+$ and $CD16^-CD56^-$ NK cells are more prevalent in the periphery (113). This observation suggests that the depletion of $CD56^+$ NK cells observed during acute HIV infection may be due in part to a redistribution of these cells to different tissues. The function of $CD56^+$ NK cells changes during SIV infection, with decreased IFN-γ production in the $\alpha4\beta7^+$ subset and increased expression of the degranulation marker CD107a, indicating greater cytotoxicity, on all $CD56^+$ NK cells (175). $CD16^+$ and $CD16^-CD56^-$ NK cells also show

increased markers of cytotoxicity during chronic SIV infection, but have decreased cytokine expression (113).

KIR and MHC Class I in Immunodeficiency Virus Infection

Genetic evidence suggests that KIR-MHC class I interactions play an important role in the prognosis for HIV infection. KIR3DL1 or KIR3DS1 coexpression with their HLA-Bw4 ligand is associated with delayed progression to AIDS (176, 177), as is an increase in copy number of these KIR (178). These genotypes are furthermore implicated in the maintenance of a subset of gut mucosal NK cells in controllers (179). KIR3DS1 and ligand coexpression is also associated with decreased opportunistic infections, but not cancers, later in disease (180). Furthermore, these same genotypes have been associated with individuals who have been exposed to HIV but have not become infected, suggesting that the protective effect of these potent NK cells may extend to viral acquisition (181).

While it is surprising that both an activating and an inhibitory KIR could have the same effect, functional studies have shown that both populations expand during acute HIV infection in individuals who express the ligand for these KIRs, perhaps indicating KIR-mediated activation of these cells (182). Furthermore, KIR3DS1$^+$ NK cells suppress HIV replication more effectively and KIR3DL1$^+$ NK cells secrete more β-chemokines that inhibit viral entry in response to HIV-infected autologous ligand-expressing cells than KIR3DL1$^-$KIR3DS1$^-$ NK cells (148, 183). These results show that KIR3DS1$^+$ and KIR3DL1$^+$ NK cells from individuals expressing ligands for these KIR are selectively activated in response to HIV-infected autologous cells. One cause of KIR3DL1$^+$ NK cell activation could be that a common escape mutation in a CD8$^+$ T cell epitope bound by HLA-B*57, a KIR3DL1 ligand, prevents recognition by KIR3DL1 (184). This

subset of NK cells may therefore be activated to kill cells infected with viral variants that escape $CD8^+$ T cell responses. In rhesus macaques KIR have also been associated with NK cell function during SIV infection. The copy number for activating KIR has been associated with SIV control (185), whereas the inhibitory Mamu-KIR3DL05 has been associated with higher viral loads (186). Additionally, activating KIR2DL4 copy number has been associated with maintenance of $CD4^+$ T cells and increased IFN-γ production from NK cells during acute SIV infection (187). Together these genetic associations and functional data show that KIR-expressing subsets of NK cells have distinct functional activity during HIV and SIV infection, and may affect the degree of viral replication and rate of progression to disease.

Perhaps the most conclusive evidence for the importance of KIR-MHC class I interactions and NK cells during immunodeficiency virus infection is the detection of KIR-associated footprints in HIV, which suggest KIR-mediated immune pressure. Polymorphisms in HIV were significantly associated with KIR expression for several activating and inhibitory KIR (188). Two of the mutations were in overlapping coding regions of *Vpu* and *Env*, and when cloned into HIV, this variant was more capable of replicating in the presence of $KIR2L2^+$ NK cells than was wild-type virus. Furthermore, KIR2DL2 binding to infected cells was increased by these mutations. It should be noted that $CD8^+$ T cells may also express KIR, and mutations that cause NK cell inhibition might also inhibit $CD8^+$ T cell killing via inhibitory KIR interactions (189, 190). The effect of viral mutations upon KIR binding may extend to other KIR as well, as naturally-occuring epitope variants affect KIR2DL2 binding to HLA-Cw*0102 ligands and KIR3DL1 binding to HLA-B*57 (184, 191), such that escape from $CD8^+$ T cell responses may increase the ability of NK cells expressing these inhibitory KIR to respond to virus-infected cells. In rhesus macaques, the binding of Mamu-KIR3DL05 to its ligand, Mamu-A1*002, is also

affected by SIV peptides. Mamu-A1*002 tetramers folded with some SIV peptides, but not others, bind Mamu-KIR3DL05 (Figure 1.7) (144). Mamu-KIR3DL05 interaction with Mamu-A1*002 folded with Gag_{71-79} GY9 is strong enough to stain this KIR on primary rhesus macaque NK cells, which has not been seen with any other tetramers tested.

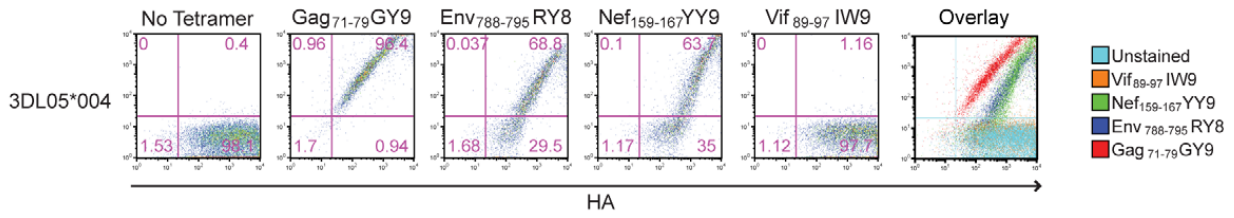


Figure 1.7. Peptide selectivity of Mamu-KIR3DL05 binding to Mamu-A1*002. Jurkat cells transfected with an HA-tagged Mamu-KIR3DL05 construct were stained with Mamu-A1*002 tetramer folded with different SIVmac239 $CD8^+$ T cell epitopes. Mamu-A1*002 tetramers folded with Gag_{71-79} GY9, more weakly to tetramers folded with $Nef_{159-167}$ YY9 or $Env_{788-795}$ RY8, and did not bind Mamu-A1*002 folded with Vif_{89-97} IW9. Reprinted under the Creative Commons Public Domain from (144).

1.4 - Scope of this Dissertation

This dissertation develops a further understanding of KIR-MHC class I interactions in the rhesus macaque. Study of rhesus macaque KIR and NK cell populations is severely restricted because few KIR ligands have been identified and few staining reagents are available to identify KIR-expressing NK cell subsets. In the second chapter of this dissertation, I have identified four Bw4 molecule ligands of the inhibitory Mamu-KIR3DL01. Mutational analysis revealed that many of the contact residues are maintained between human KIR3DL1 and Mamu-KIR3DL01 recognition, despite the lack of an orthologous relationship between these KIR. This work has also identified the binding specificity of a pan-human-KIR2D antibody that stains most allotypes of Mamu-KIR3DL01, thereby doubling the number of staining reagents available for specific rhesus macaque KIRs and enabling further studies of the frequency of KIR-expressing NK cell populations in vivo.

In the third chapter of this dissertation, I have investigated the functional relevance of the peptide preference of Mamu-KIR3DL05 recognition of Mamu-A1*002. A cytotoxicity assay was developed to evaluate Mamu-KIR3DL05^{+} NK cell killing of peptide-pulsed cells that express distinct Mamu-A1*002-peptide complexes on the cell surface. This assay enabled study of NK cell inhibition in response to cells expressing the same MHC class I molecule but presenting different peptides. Approximately one-third of the previously identified SIV peptides that bind Mamu-A1*002 inhibited Mamu-KIR3DL05^{+} NK cell activity. Peptide variants revealed that bulky residues at position 7 or 8 of the Mamu-A1*002-presented peptide could abrogate inhibition of Mamu-KIR3DL05^{+} NK cells and that suppression of this NK cell population by an inhibitory peptide is dominant over a non-inhibitory peptide when presented as part of a peptide mixture. These results, together with the diminished ability of Mamu-KIR3DL05^{+} NK cells to suppress viral replication in autologous cells from Mamu-A1*002^{+} animals, suggests that such inhibitory epitopes may evade the immune response of the Mamu-KIR3DL05^{+} NK cell subset.

Chapter 2

KIR3DL01 Recognition of Bw4 Ligands in the Rhesus Macaque: Maintenance of Bw4 Specificity since the Divergence of Apes and Old World Monkeys

2.1 - Attributions and Acknowledgements

The data presented in this chapter are reproduced with permission from:
Schafer, J. L., A. D. Colantonio, W. J. Neidermyer, D. M. Dudley, M. Connole, D. H. O'Connor, and D. T. Evans. 2014. KIR3DL01 Recognition of Bw4 Ligands in the Rhesus Macaque: Maintenance of Bw4 Specificity since the Divergence of Apes and Old World Monkeys. *J Immunol*. 192:1907-1917. Copyright 2014. The American Association of Immunologists, Inc.

JLS, ADC, and DTE designed research; JLS, ADC, and WJN performed research; DMD and DHO provided KIR typing by deep sequencing; MC sorted NK cells by FACS. JLS generated data shown in Figures 2.3-2.7; ADC and WJN generated data shown in Figures 2.1-2.3.

We are grateful to Dean Lee from the University of Texas for providing the K562 Clone 9.mbIL21 cells, and to Jacqueline Gillis, Fay Eng, and Yi Yu at the New England Primate Research Center for flow cytometry services.

This research was supported by Public Health Service grants AI095098 and AI098485 to DTE, and RR021745 to DHO. Additional support was provided by PHS grants OD011103 and OD010849. DTE is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

2.2 - Abstract

The identification of MHC class I ligands for rhesus macaque KIRs is fundamental to our basic understanding of KIR and MHC class I co-evolution and to the study of NK cell responses in this non-human primate model for AIDS and other viral diseases. Here we show that Mamu-KIR3DL01, which is expressed by approximately 90% of rhesus macaques, recognizes MHC class I molecules with a Bw4 motif. Primary NK cells expressing Mamu-KIR3DL01 were identified by staining with a mAb herein shown to bind Mamu-KIR3DL01 allotypes with an aspartic acid at position 233. The cytolytic activity of Mamu-KIR3DL01^{+} NK cells was suppressed by cell lines expressing the Bw4 molecules Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01. The Bw4 motif was necessary for Mamu-KIR3DL01 recognition, since substitutions in this region abrogated Mamu-KIR3DL01^{+} NK cell inhibition. However, the presence of a Bw4 motif was not sufficient for recognition, since another Bw4 molecule, Mamu-B*017:01, failed to suppress the cytolytic activity of these NK cells. Replacement of three residues in Mamu-B*017:01, predicted to be KIR-contacts based on the 3-dimensional structure of the human KIR3DL1-HLA-Bw4 complex, with the corresponding residues at these positions for the other Mamu-Bw4 ligands restored Mamu-KIR3DL01^{+} NK cell inhibition. These results define the ligand specificity of one of the most polymorphic and commonly expressed KIRs in the rhesus macaque, and reveal similarities in Bw4 recognition by Mamu-KIR3DL01 and human KIR3DL1, despite the absence of an orthologous relationship between these two KIRs or conservation of surface residues predicted to interact with MHC class I ligands.

2.3 - Introduction

Natural killer (NK) cells are able to recognize and kill virus-infected cells and tumor cells without prior antigenic stimulation, and therefore constitute an important innate cellular defense against infectious diseases and cancers. NK cell responses in primates are regulated in part through interactions between two highly polymorphic molecules, the killer-cell Ig-like receptors (KIRs) on NK cells and their major histocompatibility complex (MHC) class I ligands on target cells. Depending on sequences in their transmembrane and cytoplasmic domains, KIRs can transduce either inhibitory or activating signals. In the case of inhibitory KIRs, NK cell activation is suppressed upon receptor engagement of MHC class I ligands on the surface of healthy cells. Thus, NK cells bearing inhibitory KIR may become activated upon disruption of ligand recognition, either as a consequence of MHC class I downregulation by viral infection (13, 68, 98, 192, 193), deletion of *MHC class I* genes during tumor progression (134), or MHC class I presentation of antagonistic peptides (157).

Genetic evidence suggests that *KIR* and *MHC class I* polymorphisms play a significant role in determining the course of infection for several human pathogens, including hepatitis C virus (194), hepatitis B virus (195, 196), human papilloma virus (197, 198), herpes simplex virus (199), and human immunodeficiency virus (176, 177). However, studies addressing the functional implications of these observations have been limited by the lack of a suitable animal model. Mice and other rodents do not express KIRs, but instead use C-type lectin-like molecules encoded by the *Ly49* genes as polymorphic NK cell receptors for MHC class I ligands (125). Moreover, KIRs appear to be evolving at a particularly rapid pace in primates (200-203). As a consequence, there is little conservation among the *KIR* genes of different species, and it is not

possible to predict the specificity of KIR-MHC class I interactions on the basis of sequence comparisons with human KIRs.

The rhesus macaque is an important animal model for acquired immunodeficiency syndrome research (9), and for other viral diseases caused by Epstein-Barr virus (204), cytomegalovirus (205), and Kaposi's sarcoma-associated herpesvirus (206). Immunogenetic characterization of this species has also contributed to our basic understanding of the co-evolution of *KIR* and *MHC class I* genes. Rhesus macaques have duplicated *Mamu-A* and *-B* genes, which correspond to *HLA-A* and *-B* in humans (207, 208). However, they do not have a *C* locus, since *HLA-C* represents a duplication of an ancestral *B* gene that occurred after the divergence of apes and Old World monkeys (207, 208). There are as many as four *Mamu-A* genes and an undefined and variable number of *Mamu-B* genes on any given haplotype in the rhesus macaque (209, 210). In accordance with *KIR* and *MHC class I* co-evolution, macaques lack lineage III *KIR* genes, which encode KIR2DL/S specific for HLA-C (211, 212), and instead have an expanded repertoire of *KIR3DL/S* genes characterized by extensive polymorphism (203, 211-214). Indeed, 19 distinct *KIR3DL/S* genes have been identified in rhesus macaques (213, 215).

The recognition of MHC class I molecules by human KIRs is primarily determined by sequences in the ligand α1 and α2 domains. All HLA-B molecules, and some HLA-A molecules, can be classified as either Bw4 or Bw6 allotypes on the basis of residues at positions 77-83 in the α1 domain (216). KIR3DL1 is the most polymorphic human KIR and recognizes diverse HLA class I ligands that share a Bw4 motif (141). The contribution of Bw4 residues to ligand recognition by KIR3DL1 was recently corroborated by a crystal structure of KIR3DL1*001 in complex with HLA-B*5701, which revealed multiple contacts between the D1 domain of

KIR3DL1*001 and Bw4 residues of HLA-B*5701 (152). This structure also revealed additional contacts between the D1 and D2 domains of KIR3DL1*001 and the α1 and α2 domains of HLA-B*5701, indicating that the Bw4 motif is not the sole determinant of ligand recognition (152).

Only a few MHC class I ligands have been defined for non-human primate KIRs. In the rhesus macaque, these include the identification of Mamu-A1*002 as a ligand for Mamu-KIR3DL05 by binding and cellular assays (143, 144), with additional binding partners for Mamu-KIR3DL05, -3DLW03, -3DL11 and -3DS05 revealed by staining with soluble KIR-Fc fusion proteins (143). In the pig-tailed macaque, Mane-A1*082 and -A1*084 were also identified as ligands for KIR049-4 (217). However, the value of most of these interactions for studying NK cell responses in *KIR*- and *MHC class I*-defined animals remains limited due to the lack of reagents for staining specific KIR on primary macaque NK cells.

Here we show that a monoclonal antibody (mAb) to human KIR2D molecules binds allotypes of Mamu-KIR3DL01 bearing an aspartic acid at position 233. We use this Ab to define the ligand specificity of Mamu-KIR3DL01, a commonly expressed and highly polymorphic KIR in the rhesus macaque. Our results reveal similarities in the recognition of Bw4 molecules by Mamu-KIR3DL01 and human KIR3DL1, despite the absence of an orthologous relationship between these two KIRs. These observations provide an important foundation for studying NK cell responses in *KIR*- and *MHC class I*-defined rhesus macaques and reveal an ancient origin for Bw4 specificity prior to the divergence of apes and Old World monkeys.

2.4 - Materials and Methods

Ethics statement

Indian origin rhesus macaques (*Macaca mulatta*) were used in this study. Housing and care of the animals at the New England Primate Research Center (NEPRC) were in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Animal experiments were approved by the Harvard Medical Area Standing Committee on Animals and conducted according to the principles described in the *Guide for the Care and Use of Laboratory Animals* (218).

Phenotyping of NKVFS1$^+$ lymphocytes

Unless otherwise noted, all antibodies are from BD Biosciences. PBMC were isolated by Ficoll-Paque (GE Healthcare) and stained with anti-CD3-V500 (clone SP34-2), anti-NKG2A-Pacific Blue (clone Z199, in house conjugation), anti-CD4-FITC (clone L200), anti-HLA-DR-TexasRed (clone Immu-357; Beckman Coulter), anti-CD20-PE-Cy5.5 (clone L27), anti-CD56-PE-Cy7 (clone B159), anti-KIR2D-APC (clone NKVFS1; Miltenyi Biotec), anti-CD8-Alexa700 (clone RPA-T8), anti-CD16-APC-Cy7 (clone 3G8), and anti-CD14-PE (clone MφP9) or anti-NKG2D-PE (clone BAT221; Miltenyi Biotec) for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 2 x 10^5 lymphocyte events were collected using an LSRII flow cytometer (BD Biosciences), and the data was analyzed using FlowJo 8.8.7 (Tree Star).

Electroporation of Jurkat cells with KIR-expression constructs

Jurkat cells (10^7 cells) were electroporated (250 V, 950 μF) with 40 μg of plasmid DNA encoding hemagglutinin (HA)-tagged KIR in the pCGCG vector in 350 μL of RPMI 1640 (Invitrogen) in a 0.4 cm cuvette using the GenePulser ShockPod system (Bio-Rad). Cells were rested for ten minutes, then cultured overnight in RPMI 1640 supplemented with 10% FBS (Invitrogen), HEPES, and glutamine (Invitrogen). Twenty-four hours later, cells were stained with anti-HA-PE (clone GG8-IF3.3; Miltenyi Biotec) and anti-KIR2D-APC for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 3 x 10^5 events were collected using a FACSCalibur flow cytometer, and the data was analyzed, using FlowJo 8.8.7, after gating on GFP^+ cells as the transfected subset.

721.221 stable cell lines

Rhesus macaque MHC class I cDNAs were cloned into pQCXIP or pQCXIN retroviral vectors (BD Clontech). These vector were packaged into VSV-G pseudotyped MLV-based particles by cotransfection with pVSV-G (BD Clontech) into GP2-293 cells (BD Clontech). Supernatant was collected from transfected GP2-293 cells two days post-transfection and concentrated by centrifugation in Ultracel 50k filter centrifuge tubes (Millipore). 721.221 cells were transduced by incubation with concentrated virus for 3 hours at 37˚C. Three days later, cells were placed under selection with 0.4 ug/mL puromycin (Invitrogen) or 500 ug/mL of G418 (Calbiochem). Surface expression of transduced MHC class I molecules was verified by flow cytometry staining with a PE-conjugated pan-MHC class I-specific mAb (clone W6/32; DakoCytomation).

NK cell expansion and cell culture

Peripheral blood mononuclear cells (PBMC; 5 x 10^6 cells) were cultured with 1 x 10^7 γ-irradiated K562 Clone 9.mbIL21 cells (219) in a volume of 40 mL of RPMI 1640 (Invitrogen) supplemented with 10% FBS, glutamine, primocin (InvivoGen), and 50 U/mL IL-2 (AIDS Research and Reference Reagent Program). On days 3 and 5 after stimulation, cells were resuspended in fresh medium. The expanded NK cells were re-stimulated on day 7, and weekly thereafter with additional γ-irradiated K562 Clone 9.mbIL21 cells at a 1:1 ratio. From day 7 onward, expanded cells were maintained at 4 x 10^5 cells/mL by resuspension in fresh medium 2-3 times weekly.

NK cell sorting

T cells were depleted from expanded NK cell cultures by incubation with anti-CD3 Ab (clone 6G12) followed by immunomagnetic bead depletion with pan-mouse IgG Dynabeads (Dynal Biotech). Mamu-KIR3DL01$^+$ and -KIR3DL01$^-$ subsets were separated by FACS using a pan-KIR2D-specific mAb (clone NKVFS1) that cross-reacts with a subset of Mamu-KIR3DL01 allotypes. NK cells were stained with anti-CD3-Pacific Blue (clone SP34-2; BD Biosciences), anti-NKG2A-APC (clone Z199; Beckman Coulter), and anti-KIR2D-FITC (clone NKVFS1; AbD Serotec) or anti-NKG2A-Pacific Blue, anti-CD3-FITC (clone SP34; BD Biosciences), and NKVFS1-APC. NKVFS1$^+$CD3$^-$NKG2A$^+$ and NKVFS1$^-$CD3$^-$NKG2A$^+$ subsets were sorted using a FACSAria (BD Biosciences). These sorted NK cells subsets were maintained as described above by re-stimulation with γ-irradiated K562 Clone 9.mbIL21 cells.

CD107a degranulation assay

PBMC ($1x10^6$ cells) were stimulated for 6 hours with parental 721.221 cells, or with 721.221 cells that constitutively express rhesus macaque MHC class I molecules, at a 5:1 ratio in the presence of anti-CD107a-PE-Cy5 (clone H4A3; BD Biosciences), GolgiStop, and GolgiPlug (BD Biosciences). The cells were then stained with anti-CD3-FITC, anti-NKG2A-PE, and NKVFS1-APC for 20 min at 25°C. Samples were washed and fixed in 2% paraformaldehyde PBS. At least 2 x 10^5 lymphocyte events were collected using a FACSCalibur flow cytometer, and the data was analyzed using FlowJo 8.8.7.

Calcein acetoxymethyl ester cytotoxicity assay

Parental 721.221 cells or 721.221 cells expressing rhesus macaque MHC class I molecules were stained with calcein acetoxymethyl ester (CAM; Invitrogen) at a 1:100 dilution for 1 hr at 37°C. Cells were washed and then incubated with KIR3DL01$^+$ or KIR3DL01$^-$ NK cells for 4 hours at E:T ratios between 0.5:1 and 10:1. The release of CAM into the supernatant was measured using a fluorescent plate reader (excitation 485 nm, absorption 530 nm). Percent specific lysis was calculated as (test release – spontaneous release) / (maximum release – spontaneous release).

KIR *sequencing*

Total RNA was isolated from PBMC using an AllPrep DNA/RNA mini kit (Qiagen). cDNA was synthesized using a Superscript III cDNA first strand synthesis system using Oligo(dT) primers (Invitrogen). cDNA was PCR amplified using Phusion high fidelity DNA polymerase (New England Biolabs) with two primer sets for each animal. One primer set

produced a 669bp amplicon containing Roche/454 titanium adaptors and multiplex identifier tags attached to the following sequence-specific primer sequences: KIR-405-F 5'-AGGTCCCCTGGTGAAATCAG-3' and KIR-1004-R 5'-CTTGGTTCAGTGGGTGAAGG-3'. The PCR conditions for this amplicon were previously published (220). These amplicons were purified using Agencourt AMPure XP size selection beads (Beckman Coulter), quantitated using picogreen dye (Qubit), and pooled at equimolar ratios. The pool was amplified using emulsion PCR, the emulsions broken, and beads containing DNA with both adaptors were enriched and sequenced on a Roche/454 GS Junior using Titanium technology and standard protocols provided with the sequencing kits.

The other primer set amplified the full-length *KIR* coding sequence with a variety of expected sizes up to 1500bp, depending upon the *KIR* alleles within the animal, with the following sequence-specific primer sequences: KIR5'UTR.3 5'-CAGCACCATGTCGCTCAT-3' and KIR3'UTR-C 5'-GGGGTCAAGTGAAGTGGAGA-3'. The full-length *KIR* alleles were amplified with Phusion high fidelity DNA polymerase (New England Biolabs) under the following conditions: 98°C for 30 s, 28 cycles of 98°C for 5 s, 60°C for 10 s, and 72°C for 30 s, followed by a 5 min extension at 72°C. These amplicons were purified using AMPure XP beads and fragmented using Nextera XT tagmentation (Illumina), which leaves a unique Illumina-specific index identifier associated with each sample. Fragment size was determined by bioanalysis (Agilent-high sensitivity chip) and concentration determined by picogreen dye. Normalized samples were pooled together, denatured, and run on an Illumina MiSeq system using a 500 cycle MiSeq cartridge.

KIR *genotype analysis*

The Roche/454 sequences were trimmed for quality (error probability limit of 0.01) and then reads >300bp were de novo assembled with 100% minimum overlap identity and 0% mismatches per read using Geneious version 6.1 (221, 222). Consensus sequences generated from the assembly were trimmed with an error probability limit of 0.0001, 0 low quality bases, 0 maximum ambiguities and trimmed for primer sequences. These sequences were again de novo assembled at 99% minimum overlap identity and 1% maximum mismatches per read and consensus sequences were trimmed with the parameters above. These consensus sequences representing each allele within an animal were used as a backbone against which the MiSeq 250bp paired-end reads were mapped over multiple iterations using Geneious version 6.1 software. To begin, MiSeq generated paired-end sequences were set to pair the ends and trimmed for a quality of 0.01. Sequences >150bp were trimmed for primers and sequences >100bp were subjected to multiple iterations of assembly. The first iteration consisted of custom assembling the paired and trimmed MiSeq reads against a list of consensus sequences (backbones) representing the alleles found in each animal by the Roche/454 analysis with a minimum overlap identity of 100% over a minimum overlap of 95bp, 0% mismatches, and mapping best matches to none to avoid sequences mapping to more than one backbone. These Roche/454-based consensus sequences were elongated by the paired ends during this assembly and were then trimmed with an error probability limit of 0.0001. The elongated and trimmed consensus sequences generated from both the Roche/454 and MiSeq sequences were used as the backbone in place of the Roche/454 sequences for the next iteration of mapping the paired MiSeq sequences against. This iterative mapping continued until the *KIR* alleles no longer extended. Many alleles were extended from the 669 bp Roche/454 read length to full-length including start

and stop codons for each allele found within an animal. The final sequence assemblies were then blasted against a database of all *KIRs* published in Genbank to determine the identity of the *KIR* alleles for each animal. Allele designations were assigned by the Immuno Polymorphism Database (223).

Phylogenetic analysis of rhesus and human KIRs

Full-length amino acid sequences were aligned using MacVector 12.0.6 (MacVector). Phylogenetic trees were generated by the Neighbor-Joining method using MEGA 5.2 (224). The bootstrap method with 500 replicates was used with pairwise deletion and the Poisson correction was used in computing evolutionary distances.

2.5 - Results

A monoclonal Ab to human KIR2D stains rhesus macaque NK cells and CD8$^+$ T cells

We previously observed that a mAb to human KIR2D (clone NKVFS1) stained a peripheral blood lymphocyte population in some rhesus macaques (144). Further characterization of this population revealed that the majority of these cells express antigens typical of NK cells, including CD8, CD16, NKG2D, and NKG2A (Supplemental Figure 2.1A). The NKVFS1$^+$ population consisted of NK cells (CD3$^-$CD8$^+$NKG2A$^+$) and, to a lesser extent, CD8$^+$ T cells (CD3$^+$CD8$^+$NKG2A$^-$) (Supplemental Figure 2.1B). NKVFS1$^+$ cells were observed in each of the four NK cell subsets defined by CD16 and CD56 expression (Supplemental Figure 2.1C). These results, and the similar staining pattern observed for Mamu-KIR3DL05 on peripheral blood lymphocytes (144), suggest that NKVFS1 cross-reacts with a rhesus macaque KIR.

NKVFS1 binds to Mamu-KIR3DL01 allotypes with an aspartic acid residue at position 233 of the D2 domain

To identify the rhesus macaque KIR bound by NKVFS1, each of the *KIR* alleles cloned from an NKVFS1$^+$ animal were expressed from a construct with an HA tag at the N terminus of the D0 domain (144). Jurkat cells were transfected with these constructs and stained with NKVFS1 and a HA-specific mAb. For cells expressing Mamu-KIR3DL01*001, KIR expression correlated with NKVFS1 staining (Figure 2.1). In contrast, cells expressing another *Mamu-KIR3DL01* allele from this animal, *Mamu-KIR3DL01*002*, did not stain with NKVFS1. Since the Ig-like domains of these molecules differ by only eight amino acids (Figure 2.2A), reciprocal substitutions at each of these positions were tested to define the epitope bound by NKVFS1. An aspartic acid to histidine change at position 233 (D233H) of Mamu-KIR3DL01*001 abrogated

staining by NKVFS1, whereas substitutions at positions 71, 93/96/98, 104, 173, or 181 did not affect NKVFS1 staining (Figure 2.2B). Conversely, a histidine to aspartic acid substitution at position 233 (H233D) in Mamu-KIR3DL01*002 resulted in staining with NKVFS1. These results indicate that residue 233 of Mamu-KIR3DL01*001, predicted to be a surface residue in the D2 domain (152), differentiates allotypes of Mamu-KIR3DL01 that bind NKVFS1 from those that do not. This distinction was confirmed by positive NKVFS1 staining of cells expressing Mamu-KIR3DL01*014, *017, or *020 (Figure 2.2C), all of which have an aspartic acid at position 233.

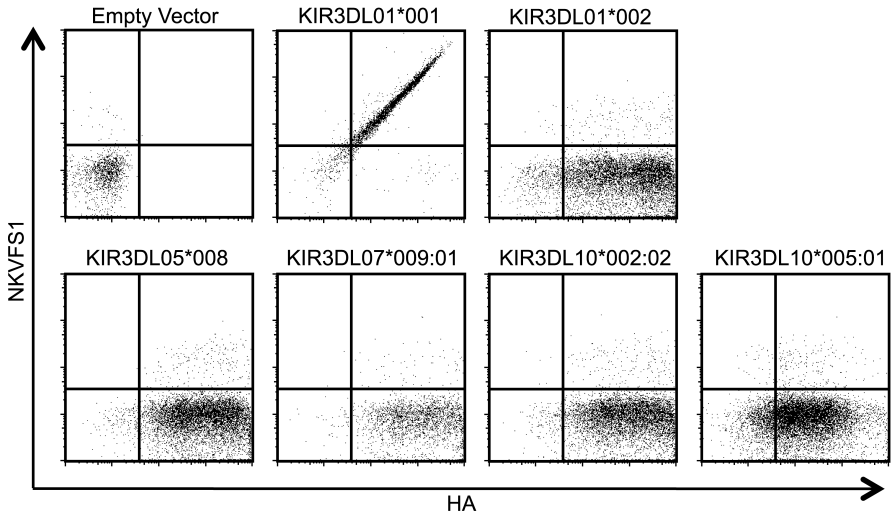


Figure 2.1. NKVFS1 stains Mamu-KIR3DL01*001. Jurkat cells were electroporated with HA-tagged KIR expression constructs that co-express GFP and were stained with the NKVFS1 Ab and an HA-specific mAb. Samples were gated on GFP^+ cells and analyzed for NKVFS1 versus anti-HA staining. Data shown is representative of results obtained in three independent experiments.

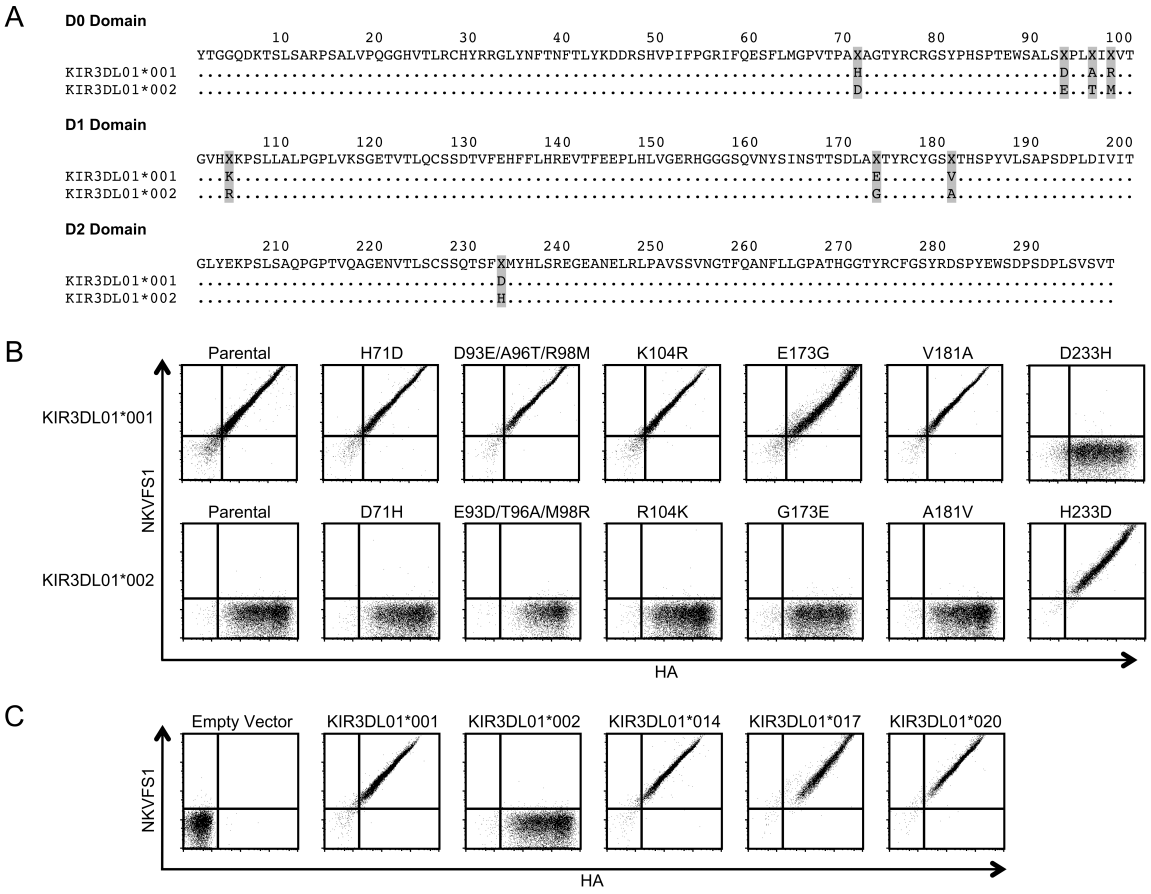


Figure 2.2. Residue 233D is essential for NKVFS1 binding to Mamu-KIR3DL01*001. (**A**) An amino acid alignment shows residues that differ between Mamu-KIR3DL01*001 and -KIR3DL01*002 in D0, D1, and D2. (**B**) Jurkat cells were electroporated with constructs expressing HA-tagged KIR with reciprocal substitutions at positions that differ between Mamu-KIR3DL01*001 and -KIR3DL01*002, or (**C**) constructs expressing five different *Mamu-KIR3DL01* alleles. Staining of GFP^+ cells for HA and NKVFS1 is shown. Data shown is representative of results obtained in three independent experiments.

Table 2.1. *Mamu-KIR3DL01* alleles expressed by the rhesus macaques in this study.

Animal	***Mamu-KIR3DL01* allele**	**Residues 230-236**
1	**001*	TSF**D**MYH
	**016*	TSF**D**MYH
2	**019*	TSF**D**MYH
3	**019*	TSF**D**MYH
4	**015*	TSF**D**MYH
5	**019*	TSF**D**MYH

The residue indicated in bold is the aspartic acid at position 233 of Mamu-KIR3DL01 that is necessary for binding by the mAb NKVFS1.

Identification of MHC class I ligands for Mamu-KIR3DL01

Having identified Mamu-KIR3DL01 D233 allotypes as the surface antigen bound by NKVFS1, we next sought to define the MHC class I ligands recognized by this KIR. The NKVFS1 Ab was used to identify additional rhesus macaques expressing Mamu-KIR3DL01 D233, and the presence of alleles coding for this KIR was confirmed by deep sequencing (Table 2.1). PBMC from four Mamu-KIR3DL01^{+} macaques were stimulated with MHC class I-deficient 721.221 cells (225), or 721.221 cells that constitutively express rhesus macaque MHC class I molecules, and stained for CD107a as a degranulation marker. The frequency of NK cells with surface CD107a was compared between Mamu-KIR3DL01^{+} (NKVFS1^{+}) and -KIR3DL01^{-} subsets from the same animal. Whereas a similar percentage of Mamu-KIR3DL01^{+} and -KIR3DL01^{-} NK cells degranulated in response to parental 721.221 cells and 721.221 cells expressing Mamu-A1*001:01, -B*017:01, -B*022:01 and -B*056:01, CD107a upregulation by the Mamu-KIR3DL01^{+} subset was selectively suppressed by incubation with 721.221 cells expressing Mamu-B*007:01-, -B*041:01, -B*058:02, and -B*065:01 (Figure 2.3A). This result was reproducible using NK cells from four unrelated animals (Figure 2.3B).

To corroborate these results, NK cell recognition of 721.221 cells expressing rhesus macaque MHC class I molecules was evaluated in cytotoxicity assays. Primary NK cells were expanded by stimulation with γ-irradiated K562 Clone 9.mbIL21 cells, which express CD64 (FcγRI), CD86 (B7-2), CD137L (4-1BBL), truncated CD19, and membrane-bound IL-21 (226). Expanded NK cells were sorted into Mamu-KIR3DL01^{+} and -KIR3DL01^{-} subsets by staining with the NKVFS1 Ab and were used as effector cells in cytotoxicity assays with 721.221 cells expressing rhesus MHC class I molecules as target cells. The 721.221 target cells were labeled with calcein acetomethyl ester (CAM), a cell-permeable dye that is converted to fluorescent

calcein by cellular esterases (227, 228), and incubated with NK cells for four hours. The release of CAM into the supernatant was measured using a fluorescent plate reader to calculate the percent specific lysis. The Mamu-KIR3DL01$^-$ NK cells lysed all of the 721.221 cell lines regardless of MHC class I expression. However, the cytolytic activity of the Mamu-KIR3DL01$^+$ NK cells was inhibited by certain MHC class I molecules. Whereas 721.221 cells expressing Mamu-A1*001:01, -B*017:01, or -B*056:01 were lysed as efficiently as parental 721.221 cells by Mamu-KIR3DL01$^+$ NK cells, target cells expressing Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 inhibited lysis by Mamu-KIR3DL01$^+$ NK cells (Figure 2.3C). The intermediate inhibition shown with target cells expressing Mamu-B*022:01 was only observed with NK cells from two of three animals. In contrast, the inhibition of Mamu-KIR3DL01$^+$ NK cells by Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 was reproducible with NK cells from three animals (Figure 2.3D). These data corroborate results obtained by CD107a staining and define these four molecules as ligands for Mamu-KIR3DL01.

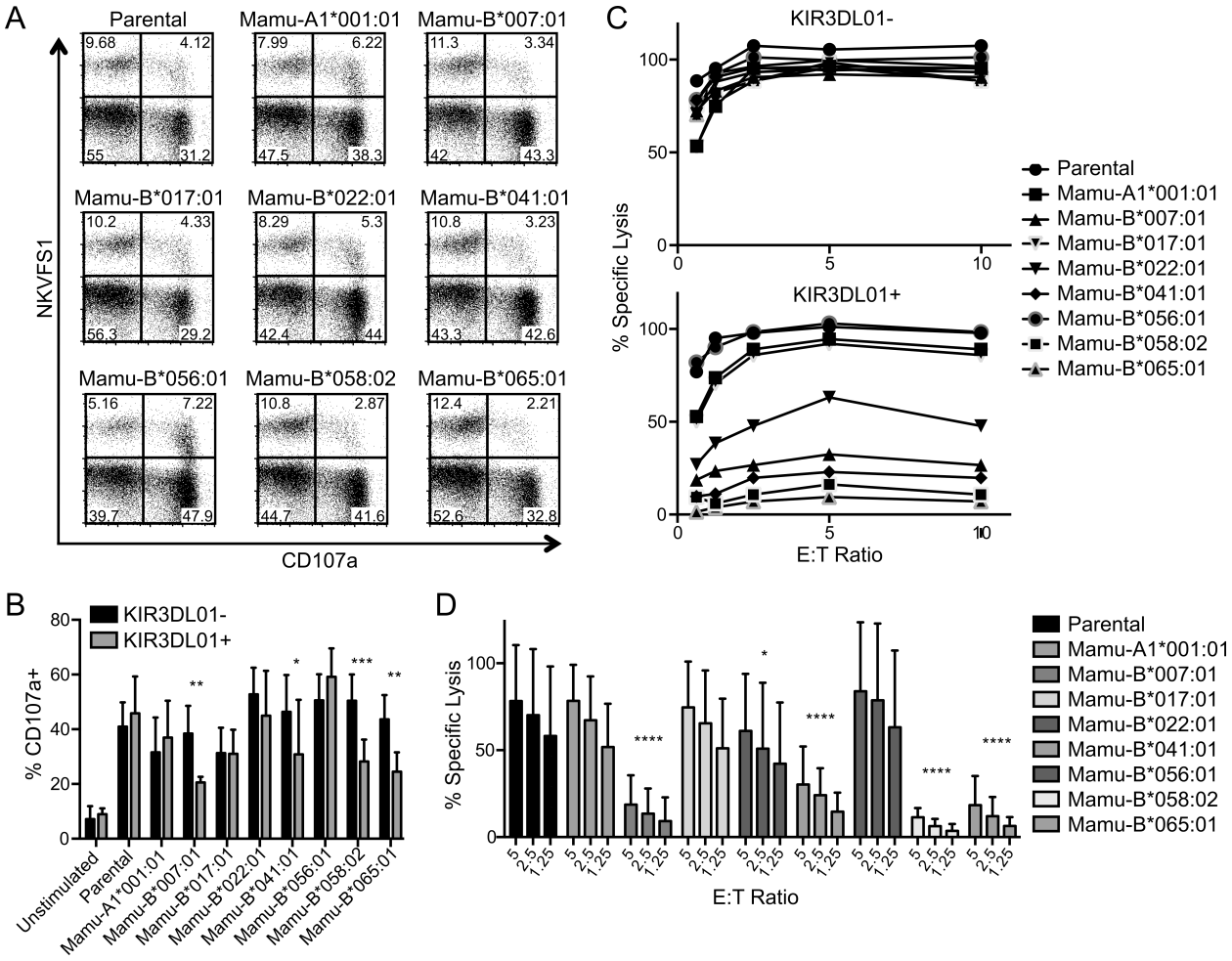


Figure 2.3. Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 are ligands for Mamu-KIR3DL01. (**A**) PBMC were stimulated overnight with parental 721.221 cells, or 721.221 cells expressing the indicated MHC class I molecules at a 5:1 ratio in the presence of a mAb to CD107a, and stained with NKVFS1 and antibodies to CD3 and CD8. After gating on $CD3^{-}CD8^{+}$ lymphocytes, the upregulation of CD107a on $NKVFS1^{+}$ versus $NKVFS1^{-}$ NK cell subsets was assessed. (**B**) The frequency of CD107a upregulation for Mamu-$KIR3DL01^{+}$ and -$KIR3DL01^{-}$ populations is summarized for four different animals where error bars indicate + 1 SD. Asterisks indicate a significant difference (*p<0.05, **p<0.01, ***p<0.005 by two-way ANOVA with Dunnett's test) between Mamu-$KIR3DL01^{+}$ and -$KIR3DL01^{-}$ populations coincubated with a given 721.221 cell line. (**C**) Mamu-$KIR3DL01^{+}$ and -$KIR3DL01^{-}$ NK cells from the same animal were coincubated with parental 721.221 cells or 721.221 cells expressing the indicated rhesus macaque MHC class I molecules at the indicated effector:target (E:T) ratios. Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. % Specific Lysis is defined as (test release – spontaneous release) / (maximum release – spontaneous release). Results are representative of those obtained with expanded cells from three different animals and the compiled results for Mamu-$KIR3DL01^{+}$ NK cells from these animals are presented in (**D**). Error bars indicate + 1 SD. Asterisks indicate a significant difference (*p<0.05, ****p<0.001 by two-way ANOVA with Dunnett's test) between coincubation with 721.221 parental cells and coincubation with the indicated cell line at all E:T ratios shown.

Residues in the Bw4 epitope contribute to recognition by Mamu-KIR3DL01

Since the rhesus macaque MHC class I molecules identified as ligands for Mamu-KIR3DL01 all have a Bw4 motif at positions 77-83 in the α1 domain (216), substitutions were introduced into this sequence to determine if the Bw4 motif contributes to recognition by Mamu-KIR3DL01. Mamu-B*065:01 residues at positions that differ between Bw4 and Bw6 motifs (77, 80, 81, 82, and 83) were exchanged, separately and in combination, with the corresponding Bw6 residues at these positions. 721.221 cells expressing these Mamu-B*065:01 mutants were tested for recognition by sorted primary Mamu-KIR3DL01^{+} and -KIR3DL01^{-} NK cells from the same animal in CAM cytotoxicity assays. Whereas none of the MHC class I mutants inhibited the cytolytic activity of the Mamu-KIR3DL01^{-} NK cells, 721.221 cells expressing Mamu-B*065 with an alanine to leucine substitution at position 81 (A81L) or a leucine to arginine substitution at position 82 (L82R) inhibited the cytolytic activity of Mamu-KIR3DL01^{+} NK cells to a similar extent as cells expressing wild-type Mamu-B*065 (Figure 2.4A). However, 721.221 cells expressing Mamu-B*065 with an asparagine to serine substitution at position 77 (N77S), a threonine to asparagine substitution at position 80 (T80N), an arginine to glycine substitution at position 83 (R83G), or Bw6 residues at each of the five positions (Mamu-B*065-Bw6), were susceptible to lysis by Mamu-KIR3DL01^{+} NK cells (Figure 2.4A). The susceptibility of cell lines expressing Mamu-B*065-N77S, -T80N, -R83G, or -Bw6 to lysis by Mamu-KIR3DL01^{+} NK cells was reproducible using primary NK cells isolated from three unrelated animals (Figure 2.4B). Thus, residues 77, 80, and 83 within the Bw4 epitope contribute to ligand recognition by Mamu-KIR3DL01.

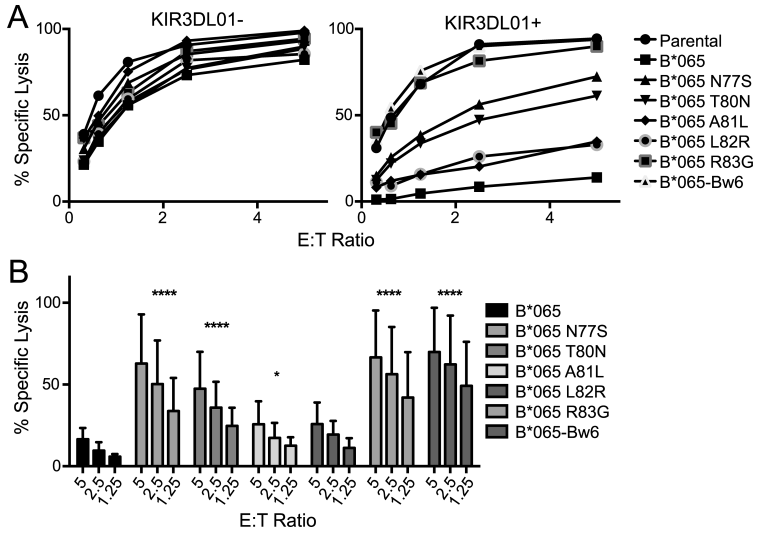


Figure 2.4. Mamu-KIR3DL01 recognition of Mamu-B*065 is dependent upon Bw4 residues N77, T80, and R83. Mutations were introduced into the Bw4 region of Mamu-B*065:01 to make it Bw6-like at positions which vary between Bw4 and Bw6. (**A**) Mamu-KIR3DL01^{+} and -KIR3DL01^{-} NK cells from the same animal were coincubated with parental 721.221 cells or 721.221 cells expressing the indicated Mamu-B*065:01 mutants at the indicated E:T ratios. Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. Results are representative of expanded NK cells from three different animals, and the compiled results for Mamu-KIR3DL01^{+} NK cells from these animals are presented in (**B**) wherein error bars indicate + 1 SD. Asterisks indicate a significant difference (*$p<0.05$, ****$p<0.001$ by two-way ANOVA with Dunnett's test) between coincubation with 721.221-Mamu-B*065 cells and coincubation with 721.221 cells expressing a given Mamu-B*065 mutant at all E:T ratios shown.

Additional MHC class I residues necessary for recognition by Mamu-KIR3DL01

Although Mamu-B*017:01 is identical to Mamu-B*007:01 and -B*065:01 at positions 77-83, Mamu-B*017:01 did not suppress the activation of Mamu-KIR3DL01^{+} NK cells, indicating that the presence of a Bw4 motif in the α1 domain is not sufficient for recognition by Mamu-KIR3DL01. To identify additional residues necessary for this interaction, reciprocal substitutions between Mamu-B*017:01 and -B*065:01 were introduced at polymorphic sites predicted to be contact residues based on the KIR3DL1-HLA-B*57 crystal structure (Figure 2.5A) (152). 721.221 cells expressing these MHC class I mutants were tested for recognition in a CAM cytotoxicity assay with sorted Mamu-KIR3DL01^{+} and -KIR3DL01^{-} NK cells from the

same animal. None of the MHC class I mutants inhibited the cytolytic activity of the Mamu-KIR3DL01$^-$ NK cells. Whereas 721.221 cells expressing wild-type Mamu-B*065:01 suppressed killing by Mamu-KIR3DL01$^+$ NK cells, substitutions in Mamu-B*065 at positions 76 (G76E), 142 (F142N), or 149 (A149G) abrogated this inhibition (Figure 2.5B). Consistent with a role for these residues in recognition by Mamu-KIR3DL01, a combination of all three reciprocal substitutions was necessary to efficiently inhibit the cytolytic activity of Mamu-KIR3DL01$^+$ NK cells in the context of Mamu-B*017:01 (Figure 2.5B). These results were reproducible with Mamu-KIR3DL01$^+$ NK cells from three different animals (Figure 2.5C). Therefore, in accordance with the three-dimensional structure of human KIR3DL1 in complex with HLA-B*57, additional residues in the α1 and α2 domains, including G76, F142, and A149, contribute to ligand recognition by Mamu-KIR3DL01.

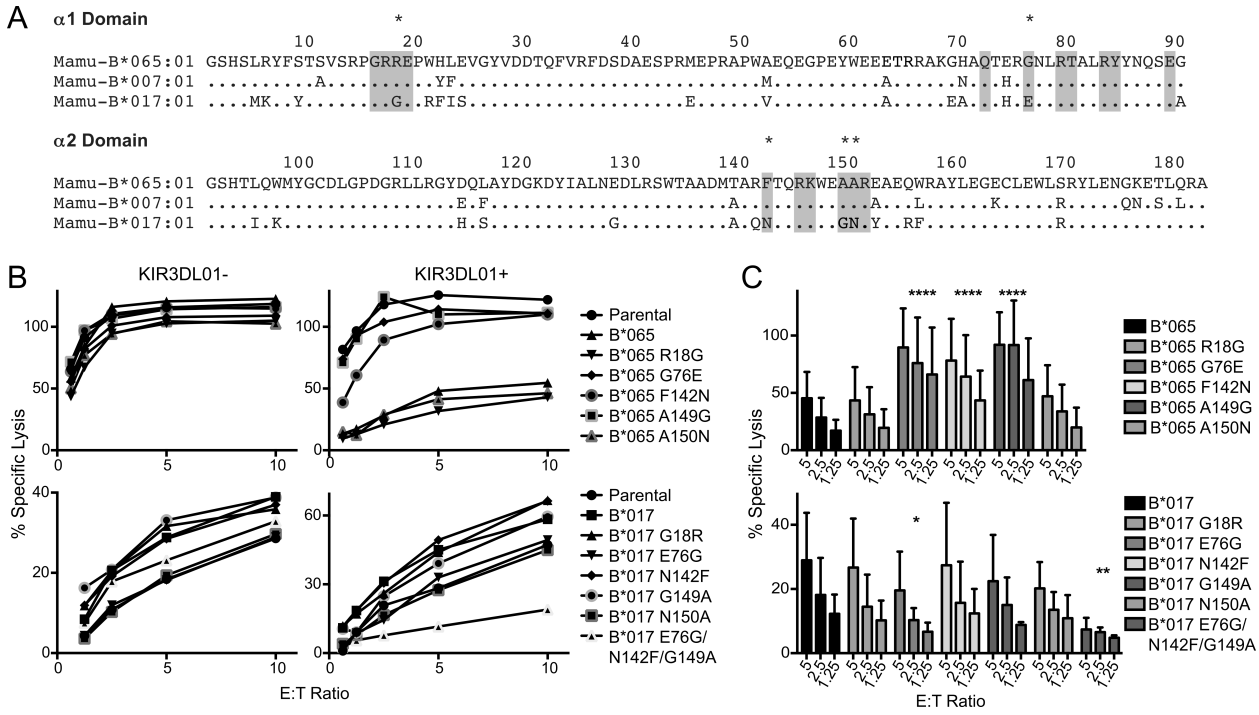


Figure 2.5. Residues G76, F142, and A149 are determinants of recognition by Mamu-KIR3DL01. (**A**) An amino acid alignment of the MHC class I α1 and α2 domains of Mamu-B*065:01, -B*007:01, and -B*017:01 with the predicted KIR contact residues highlighted based on the HLA-B*57:KIR3DL1*001 crystal structure (152). Reciprocal substitutions were introduced into Mamu-B*065:01 and -B*017:01 at each of the positions indicated with asterisks that are the same for Mamu-B*065:01 and -B*007:01, but differ for Mamu-B*017:01. (**B**) Mamu-KIR3DL01^{+} and -KIR3DL01^{-} NK cells from the same animal were coincubated with parental 721.221 cells or 721.221 cells expressing the indicated rhesus macaque MHC class I mutants at E:T ratios ranging from 10 to 0.625. Cytotoxicity was measured by the release of CAM from target cells into the culture supernatant. Results are representative of those obtained with expanded cells from three different animals, and the compiled results for Mamu-KIR3DL01^{+} NK cells from these animals are presented in (**C**), wherein error bars indicate + 1 SD. Asterisks indicate a significant difference (*$p<0.05$, **$p<0.01$, ****$p<0.001$ by two-way ANOVA with Dunnett's test) between coincubation with 721.221-Mamu-B*065:01 or -B*017:01 cells and coincubation with 721.221 cells expressing a given Mamu-B*065:01 or -B*017:01 mutant at all E:T ratios shown.

Comparison of rhesus macaque and human Bw4 ligands

Many of the residues required for Mamu-KIR3DL01 recognition of rhesus macaque Bw4 molecules are also contact residues in the human KIR3DL1:HLA-B*57 crystal structure (152). An amino acid alignment of HLA-B*57 and the seven Mamu-B molecules investigated in this study demonstrates that 13 of the 17 predicted KIR contact residues are conserved among these molecules (Figure 2.6A). Notably, residues 76 and 142 differ between HLA-B*57 and the Mamu-KIR3DL01 ligands, despite their importance for KIR recognition in both species. These residues, along with residue 149, are also polymorphic among the Mamu-B molecules studied. Whereas the four molecules identified as ligands for Mamu-KIR3DL01 have residues G76, F142, and A149, the non-ligands (Mamu-B*017:01, -B*022:01, and -B*056:01) have an asparagine at position 142 (N142), with Mamu-B*017:01 also differing at the two other positions. These three residues, and the Bw4 motif residues 77, 80, and 83, are located at surface exposed positions near the C-terminus of the α1-domain and the N-terminus of the α2-domain (Figure 2.6B), partially overlapping the footprint of KIR3DL1 bound to HLA-B*57 (152).

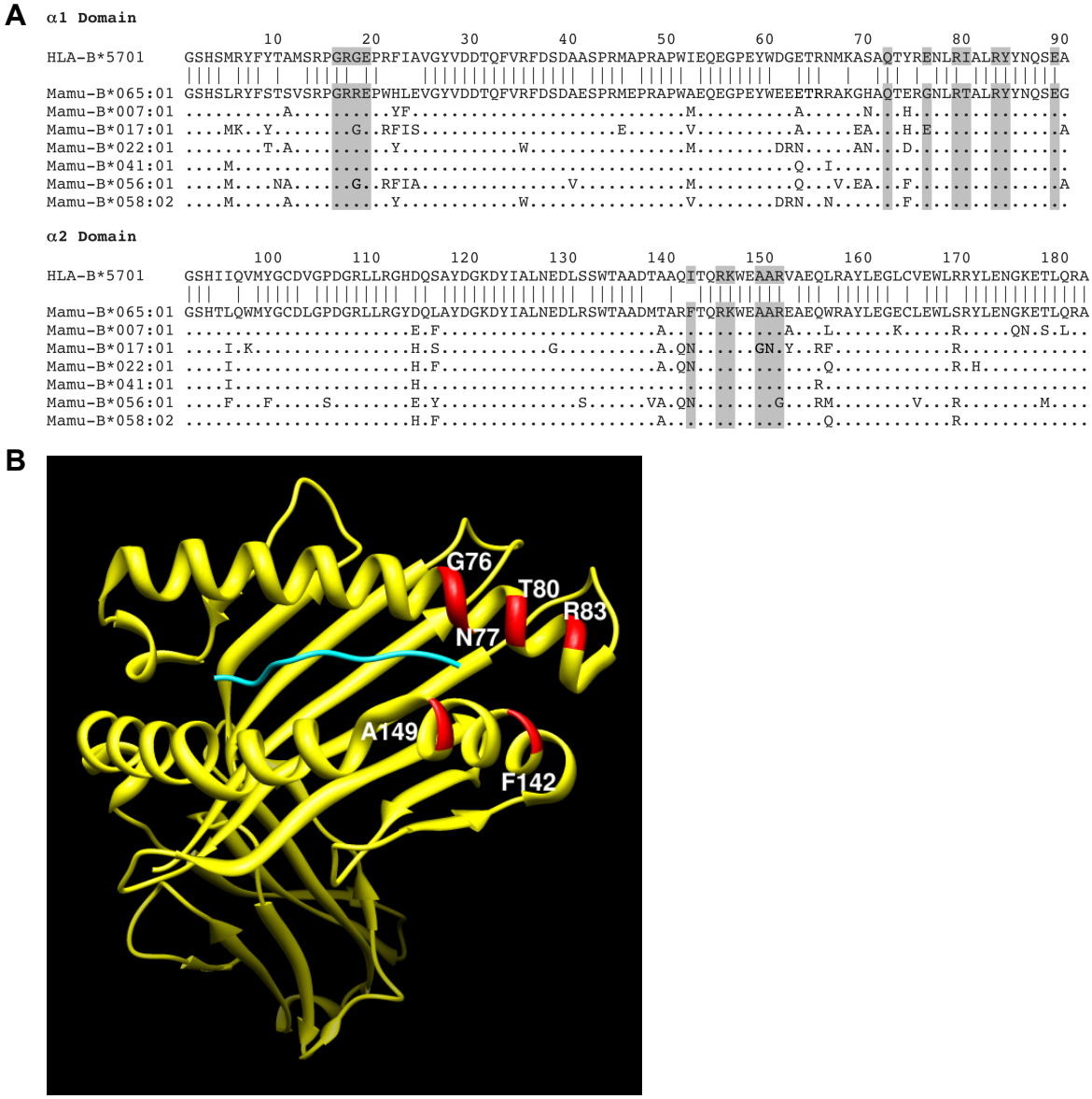


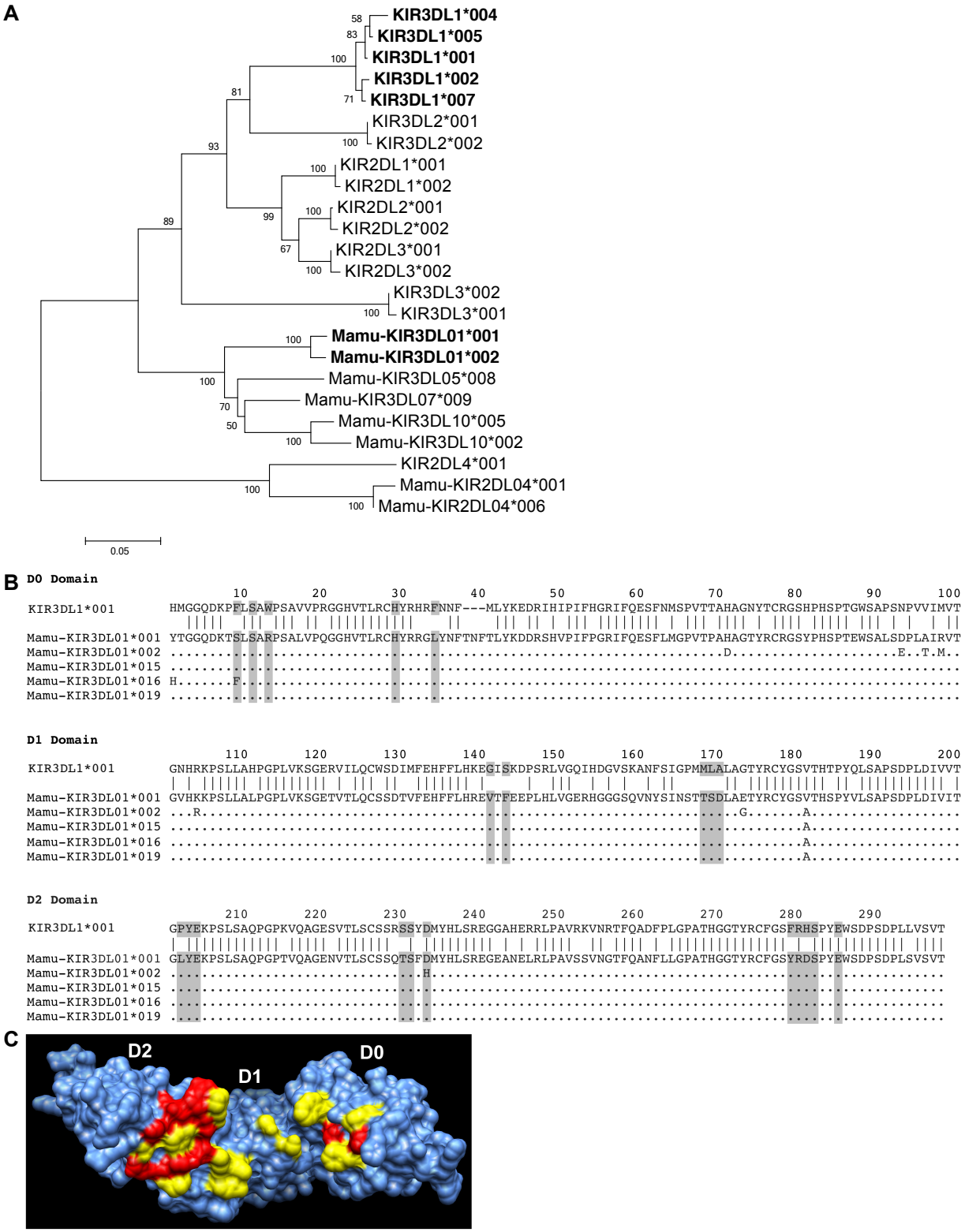
Figure 2.6. KIR contact residues of rhesus macaque Bw4 molecules. (**A**) An amino acid alignment of the MHC class I α1 and α2 domains of HLA-B*5701 and Mamu-B*065:01, -B*007:01, -B*017:01, -B*022:01, B*041:01, B*056:01, and B*058:02 with the predicted KIR contact residues shaded based on the HLA-B*57:KIR3DL1*001 crystal structure (152). (**B**) Crystal structure of HLA-B*57 (yellow) and bound peptide (cyan). Highlighted in red are positions important for recognition by Mamu-KIR3DL01 identified in this study: residues 76, 77, 80, 83, 142, and 149.

Mamu-KIR3DL01 is phylogenetically distinct from human KIR3DL1

Despite their shared specificity for Bw4 ligands and a coincidental similarity in nomenclature, Mamu-KIR3DL01 and human KIR3DL1 are not orthologous gene products. This is illustrated by phylogenetic comparisons of the predicted amino acid sequences for human and rhesus macaque KIRs. In contrast to Mamu-KIR2DL04 and human KIR2DL4, which cluster together as the most conserved KIR between humans and rhesus macaques (203), Mamu-KIR3DL01 and human KIR3DL1 are separated by deep branch lengths and are more similar to other KIRs of their respective species than they are to each other (Figure 2.7A). A nearly identical tree was obtained from an analysis that was restricted to the D1 and D2 domains, indicating that the shared specificity of Mamu-KIR3DL01 and human KIR3DL1 for Bw4 molecules is not the result of greater similarity in their ligand-binding domains (data not shown). Indeed, only 7 of the 16 residues in the D1 and D2 domains of KIR3DL1 that participate in interactions with HLA-B*57 match residues in Mamu-KIR3DL01 (Figure 2.8B). Interestingly, most of these conserved residues are in the D2 domain. Whereas 7 of the 11 HLA-contact residues of KIR3DL1 are conserved in the D2 domain of Mamu-KIR3DL01, none of 5 contacts match D1 domain residues of Mamu-KIR3DL01 (Figure 2.7B-C). Thus, the specificity of Mamu-KIR3DL01 for Bw4 ligands could not have been predicted based on sequence similarity with human KIR3DL1.

Figure 2.7. Phylogenetic analysis of human and rhesus macaque KIRs. (**A**) Midpoint-rooted phylogenetic tree generated from full-length amino acid sequences using Neighbor-Joining (NJ) method. Bootstraps were calculated with 500 iterations. (**B**) An amino acid alignment of the D0, D1, and D2 domains of human KIR3DL1 and allotypes of Mamu-KIR3DL01 with the predicted MHC ligand contact residues shaded based on the HLA-B*57:KIR3DL1*001 crystal structure (152). (**C**) Crystal structure of human KIR3DL1 with HLA-B*57 contact residues highlighted. Residues highlighted in red are conserved between KIR3DL1 and Mamu-KIR3DL01*001, whereas residues highlighted in yellow differ between the two KIRs.

Figure 2.7 (Continued).



2.6 - Discussion

The identification of MHC class I ligands for rhesus macaque KIRs is important for studying NK cell responses in this non-human primate model for AIDS and other infectious diseases. KIRs are of particular interest in the context of viruses due to genetic associations between *KIR* and *MHC class I* alleles and the ability to control certain viral infections, including HIV (176, 177). In this study, we identified a mAb that binds selectively to D233 allotypes of Mamu-KIR3DL01. Using this mAb to distinguish KIR3DL01^{+} NK cells, we identified Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 as ligands for Mamu-KIR3DL01. We also defined residues in the α1 and α2 domains of these molecules that are essential for recognition by Mamu-KIR3DL01, facilitating the prediction of additional ligands for this KIR. These results reveal similarities in the recognition of Bw4 ligands by rhesus macaque KIR3DL01 and human KIR3DL1, despite differences in the MHC class I contact residues of these receptors and the considerable divergence of *KIR* and *MHC class I* genes since these species last shared a common ancestor.

Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 are the first B molecules to be identified as KIR ligands in the rhesus macaque. As products of the extensively duplicated and highly polymorphic *Mamu-B* genes, the diversity of these molecules reflects the diversity of Mamu-KIR3DL01, which is the most polymorphic of the rhesus macaque KIRs (214). Mamu-KIR3DL01 is also one of the most commonly expressed rhesus macaque KIRs [expressed by approximately 85-95% of Indian-origin animals (213-215, 220)], and is thus likely to play a significant role in regulating NK cell responses in a majority of animals. Therefore, the identification of MHC class I ligands for Mamu-KIR3DL01 is especially important for investigating the role of NK cells in vaccine and pathogenesis studies in rhesus macaques.

The Bw4 ligands of Mamu-KIR3DL01 and human KIR3DL1 share a number of features necessary for KIR recognition. Corresponding to the importance of arginine at position 83 for HLA-Bw4 recognition by human KIR3DL1 (152, 229), this residue is also critical for Bw4 recognition by Mamu-KIR3DL01. Likewise, just as threonine or isoleucine at position 80 contributes to interactions with human KIR3DL1 (152), a threonine at this position is important for Bw4 recognition by Mamu-KIR3DL01. Similar amino acid positions outside of the Bw4 motif are also important for ligand recognition by these two KIRs. Replacement of amino acids 76, 142, or 149 of Mamu-B*065:01 with the corresponding residues from Mamu-B*017:01 impaired recognition by Mamu-KIR3DL01. Conversely, replacement of these three residues in Mamu-B*017:01 with the corresponding residues of the Mamu-Bw4 ligands conferred an interaction with Mamu-KIR3DL01. These results illustrate the similarity in Bw4 recognition by KIR3DL1 and Mamu-KIR3DL01, since these positions are also HLA-contact sites for KIR3DL1 (152).

However, there are also differences in the MHC class I residues required for recognition by Mamu-KIR3DL01 and human KIR3DL1. Whereas KIR3DL1 is dependent on leucine at position 82 (229), Mamu-KIR3DL01 recognizes ligands with leucine or arginine at this position equally well. Moreover, although position 77 is not a contact residue for KIR3DL1 (152), and substitutions at this position do not affect KIR3DL1 interactions with HLA-B*51:01 or -B*15:13 (229), position 77 is essential for Bw4 recognition by Mamu-KIR3DL01. An asparagine-to-serine substitution at this position in Mamu-B*065 abrogates its ability to inhibit Mamu-KIR3DL01^{+} NK cells. Thus, Mamu-KIR3DL01 and human KIR3DL1 have overlapping, but distinct, specificities for their respective ligands.

Although KIR recognition of Bw4 ligands has been maintained since humans and macaques last shared a common ancestor, the KIRs conferring this specificity have not. Fewer than half of the HLA-B*57 contact residues of KIR3DL1 are conserved with Mamu-KIR3DL01. Most of the differences between these receptors are in the D0 and D1 domains, which together retain only 2 of 10 MHC class I-contacts. In contrast, 7 of 11 MHC class I-contacts are retained in the D2 domain. Greater conservation of the D2 domain may reflect greater evolutionary constraint to preserve core MHC class I binding interactions, while allowing the D0 and D1 domains more freedom to fine tune ligand specificity. This possibility is consistent with structural data revealing greater complementarity in shape and charge of residues at the D2 interface (152), and the observation that polymorphisms in the D0 and D1 domains can affect the avidity and peptide-specificity of MHC class I interactions (144).

The cross-reactivity of the anti-human KIR2D mAb NKVFS1 with D233 allotypes of Mamu-KIR3DL01, which comprise 20 of the 27 defined Mamu-KIR3DL01 allotypes (223), effectively doubles the number of KIR-defined NK cell subsets that can be studied in the rhesus macaque. Although several mAbs to rhesus macaque KIRs were recently described, only one was specific for a single KIR (230). Coincidentally, this mAb bound to Mamu-KIR3DL05 (230), which can also be stained with Mamu-A1*002 tetramers (144). The availability of reagents for differentiating NK cells expressing specific KIRs is essential for dissecting the role of KIRs in primary NK cell activation *in vitro*, and for monitoring longitudinal changes of specific NK cell subsets *in vivo*. NKVFS1 will therefore be especially useful for monitoring phenotypic and functional changes in Mamu-KIR3DL01 $D233^{+}$ NK cells after experimental infection of rhesus macaques with SIV and other viral pathogens.

In summary, the present study identifies Mamu-B*007:01, -B*041:01, -B*058:02, and -B*065:01 as ligands for Mamu-KIR3DL01. Further characterization of the determinants of ligand recognition revealed that this KIR has a similar specificity for Bw4 molecules as human KIR3DL1. As one of the most common and most polymorphic KIRs in the rhesus macaque, identification of the MHC class I molecules recognized by Mamu-KIR3DL01 provides an important foundation for studying NK cell responses in this non-human primate model. These observations also suggest that, despite the divergence of *KIR* and *MHC class I* genes (231), KIR specificity for Bw4 ligands has been maintained since rhesus macaques and humans last shared a common ancestor approximately 25 million years ago (232).

Chapter 3

Modulation of Natural Killer Cell Activation by Simian Immunodeficiency Virus Peptides

3.1 - Attributions and Acknowledgements

The data in this chapter are part of a manuscript in preparation by:

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JLS and DTE designed research; JLS and NG performed research; MC sorted NK cells by FACS; EJW provided the 721.221-ICP47 cell line; NAW provided Mamu-A1*002 tetramers. JLS generated data shown in all figures. NG generated data shown in Figures 3.1-3.3 and 3.5.

We are grateful to Arnaud Colantonio for insightful comments, to Dean Lee from the University of Texas for providing the K562 Clone 9.mbIL21 cells, to Jacqueline Gillis, Fay Eng, and Yi Yu at the New England Primate Research Center for flow cytometry services, and to Valerie Varner at the New England Primate Research Center for cell irradiation services.

This research was supported by Public Health Service grants AI095098 and AI098485 to DTE. Additional support was provided by PHS grants OD011103 and OD010849. DTE is an Elizabeth Glaser Scientist of the Elizabeth Glaser Pediatric AIDS Foundation.

3.2 - Abstract

Natural killer cells recognize and kill virus-infected cells without prior antigenic stimulation, and thus provide an important innate defense against infection. NK cell responses in primates are regulated in part through interactions between two highly polymorphic molecules, the killer cell immunoglobulin-like receptors (KIRs) on NK cells and their MHC class I ligands on target cells. NK cells bearing inhibitory KIRs become activated upon disruption of MHC class I recognition. We previously reported that the binding of a common MHC class I molecule in the rhesus macaque, Mamu-A1*002, to the inhibitory receptor Mamu-KIR3DL05 is stabilized by certain SIV peptides, but not by others. In the present study, we used a Mamu-A1*002-expressing, TAP-inhibited cell line and primary Mamu-KIR3DL05^{+} NK cells to screen SIV peptides in cytotoxicity assays for the ability to modulate NK cell activation. Twenty-eight of 75 SIV peptides that bind to Mamu-A1*002 suppressed the cytolytic activity of Mamu-KIR3DL05^{+} NK cells, including three immunodominant CD8^{+} T cell epitopes previously shown to bind Mamu-A1*002. An analysis of peptide variants revealed that substitutions at positions 6, 7 or 8 could change an inhibitory peptide into a non-inhibitory peptide, and vice versa, without altering the ability to bind to Mamu-A1*002. In competition assays with mixtures of inhibitory and non-inhibitory peptides, low concentrations of inhibitory peptides dominated to suppress NK cell activation. These results demonstrate that viral peptides can modulate NK cell activation through KIR-MHC class I interactions, and are consistent with the hypothesis that immunodeficiency viruses may acquire changes in epitopes that increase the avidity of MHC class I ligands for inhibitory KIRs to suppress the activation of specific NK cell subsets.

3.3 - Introduction

Natural killer (NK) cells are an important early defense against viral infection and cancers because they can recognize and kill infected or malignant cells without prior antigenic stimulation. NK cell responses in primates are regulated by signals received from cell surface receptors, including activating and inhibitory killer-cell immunoglobulin-like receptors (KIRs). Inhibitory KIR recognition of self-MHC class I molecules on the surface of healthy cells prevents NK cell activation. The disruption of interactions between inhibitory KIRs and their MHC class I ligands, as a result of MHC class I downregulation or deletion of *MHC class I* genes, can result in NK cell killing of virus-infected (13, 68, 98, 192, 193) and tumor cells (134), respectively.

Crystal structures of KIRs in complex with ligand have revealed that although KIR binding to ligands is largely driven by residues in the α1 and α2 domains of the MHC class I molecule, contacts also exist between KIR and the MHC class I-bound peptide (145, 150-152). In fact, ligand recognition by inhibitory KIRs can be altered by the presented peptide (142, 146, 156, 157, 160, 233-236). Loss of inhibition resulting from peptides that alter the interaction between an inhibitory KIR and its MHC class I ligand can therefore lead to NK cell killing (157). This peptide-dependent NK cell regulation is of particular interest in viral infection, during which the peptide repertoire presented by infected cells changes dramatically (158, 159) and may affect binding of inhibitory KIRs to MHC class I ligands.

The importance of KIR-MHC class I interactions in viral infections has been suggested by the genetic association between KIR and MHC class I polymorphisms and the outcome of many viral infections, including hepatitis C virus (194), hepatitis B virus (195, 196), human papilloma virus (197, 198), HSV (199), and HIV (176, 177, 237). Furthermore, several HIV

polymorphisms have been detected that are associated with the expression of a specific KIR, suggesting selection by KIR-mediated immune pressure (188). HIV polymorphisms associated with KIR2DL2 expression result in increased binding of this inhibitory KIR to infected cells and a decrease in the ability of NK cells from KIR2DL2^{+} individuals to suppress viral replication (188). However, the mechanism of this inhibition remains undefined, as presentation of peptides containing these KIR-associated polymorphisms by an MHC class I KIR ligand has not been demonstrated. The potential modulation of KIR and MHC class I interactions by MHC class I-bound viral peptides during infection therefore warrants further investigation.

We previously demonstrated that the binding of a common MHC class I molecule in the rhesus macaque, Mamu-A1*002, to the inhibitory Mamu-KIR3DL05 is stabilized by certain SIV peptides, but not by others (144). To investigate the functional implications of these findings and evaluate the hypothesis that these inhibitory peptides may serve as a mechanism of immune evasion to prevent killing of virus-infected cells by a subset of NK cells, we evaluated Mamu-KIR3DL05^{+} NK cell killing of Mamu-A1*002^{+} TAP-inhibited cells pulsed with different SIV peptides. This approach allows interrogation of NK cell killing of cells that express the same MHC class I but present different peptides. Using this assay, we show that certain SIV peptides suppress the cytolytic activity of Mamu-KIR3DL05^{+} NK cells, and that the effect of an inhibitory peptide is dominant over non-inhibitory peptide when presented as part of a peptide mixture. In addition, Mamu-KIR3DL05^{+} NK cells are inhibited in the ability to suppress viral replication in Mamu-A1*002^{+} cells. These results suggest that inhibitory viral peptides may serve as a mechanism of immune evasion to prevent killing of infected cells by Mamu-KIR3DL05^{+} NK cells.

3.4 - Materials and Methods

Ethics statement

The Indian origin rhesus macaques (*Macaca mulatta*) used in this study were housed and cared for at the New England Primate Research Center (NEPRC) in accordance with standards of the Association for Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee. Animal experiments were approved by the Harvard Medical Area Standing Committee on Animals and conducted according to the principles described in the *Guide for the Care and Use of Laboratory Animals* (218).

*721.221-ICP47-A1*002 stable cell line*

Rhesus macaque *Mamu-A1*002* cDNA was cloned into the pQCXIP retroviral vector (Clontech). This vector was cotransfected with pVSV-G (Clontech) into GP2-293 cells and supernatant was harvested two days post-transfection. The supernatant was centrifuged in Ultracel 50k filter centrifuge tubes (Millipore) to yield concentrated VSV-G pseudotyped MLV-based particles. 721.221-ICP47 cells (238) were transduced by incubation with concentrated virus for 3 hours at 37˚C. Three days later, cells were placed under selection with 0.4 ug/mL puromycin (Invitrogen).

NK cell expansion and cell culture

PBMC (5×10^6 cells) were stimulated with 1×10^7 γ-irradiated K562 Clone 9.mbIL21 cells (219) in a volume of 40 mL of RPMI 1640 (Invitrogen) supplemented with 10% FBS (Invitrogen), glutamine (Invitrogen), Primocin (InvivoGen), and 50 U/mL IL-2 (AIDS Research and Reference Reagent Program). On days 3 and 5 after culture, cells were resuspended in fresh

medium. The expanded NK cells were re-stimulated on day 7, and weekly thereafter, with additional γ-irradiated K562 Clone 9.mbIL21 cells at a 1:1 ratio. From day 7 onward, expanded cells were resuspended in fresh medium at 4×10^5 cells/mL 2-3 times weekly.

NK cell sorting

Expanded NK cell cultures were incubated with anti-CD3 Ab (clone 6G12) and T cells were depleted using pan-mouse IgG Dynabeads (Dynal Biotech). Mamu-KIR3DL05$^+$ and -KIR3DL05$^-$ subsets were separated by FACS using a Mamu-A1*002 tetramer folded with $Gag_{71\text{-}79}$ GY9 that binds Mamu-KIR3DL05 (144). NK cells were stained with PE-conjugated Mamu-A1*002-GY9 tetramer for 30 minutes at 37°C followed by staining with anti-CD3-Pacific Blue (clone SP34-2; BD Biosciences) and anti-NKG2A-APC (clone Z199; Beckman Coulter) or anti-NKG2A-Pacific Blue and anti-CD3-FITC (clone SP34; BD Biosciences) for 20 minutes at 25°C. Tetramer$^+$CD3$^-$NKG2A$^+$ and Tetramer$^-$CD3$^-$NKG2A$^+$ subsets were sorted using a FACSAria (BD Biosciences). After sorting, these NK cells subsets were used immediately in a viral suppression assay or were stimulated with γ-irradiated K562 Clone 9.mbIL21 cells and maintained as described above.

Calcein acetoxymethyl ester cytotoxicity assay

Mamu-A1*002-binding SIV peptides (GenScript and Mimotopes) were pulsed onto 721.221-ICP47-A1*002 cells overnight at 26°C in Hybridoma-Serum Free Medium (Invitrogen) to stabilize cell surface Mamu-A1*002-peptide complexes. An aliquot of 2×10^5 peptide-pulsed cells were then stained with a PE-conjugated pan-MHC class I specific antibody (clone W6/32; Dako) to verify this surface stabilization. The remaining peptide-pulsed cells were stained with

calcein acetoxymethyl ester (CAM; Invitrogen) at a 1:100 dilution for 1 hr at 26°C. CAM-stained cells were washed and then incubated with Mamu-KIR3DL05^{+} or -KIR3DL05^{-} NK cells for 4 hours at E:T ratios between 0.5:1 and 10:1. The release of CAM into the supernatant was measured using a fluorescent plate reader (excitation 485 nm, absorption 530 nm). Percent specific lysis was calculated as (test release – spontaneous release) / (maximum release – spontaneous release).

Viral suppression assay

The target cells were prepared by depletion of CD8^{+} cells from PBMC by treatment with CD8-Dynabeads (Dynal Biotech) followed by ConA-activation (5 μg/ml) in RPMI 1640 supplemented with 10% FBS, glutamine, HEPES (Invitrogen), and Primocin (R10) for 4 days. On the day of the assay, 2×10^6 target cells were infected with 100 ng p27 $SIV_{mac}239$ in minimal volume for 3 hours at 37°C, then washed. Mamu-KIR3DL05^{+} and -KIR3DL05^{-} NK cell subsets were sorted as described above and were incubated with 2×10^4 SIV-infected autologous CD4^{+} target cells in 200 uL R10 + 20 U/mL IL-2 in duplicate wells at a 3:1 E:T ratio. On days 3, 5, 7, and 9/10, 50 uL of supernatant was sampled with replacement. SIV titer was determined by SIV p27 Antigen Capture Assay (Advanced Bioscience Laboratories). Area under the curve calculated in Prism 6 (GraphPad).

3.5 - Results

*Mamu-A1*002-SIV peptide complexes differentially inhibit Mamu-KIR3DL05$^+$ NK cells*

We previously demonstrated that Mamu-A1*002 SIV tetramers folded with some SIV peptides, but not others, bind Mamu-KIR3DL05 (144). To evaluate the functional implications of these interactions, we developed a peptide-pulsing cytotoxicity assay. MHC class I-deficient 721.221 target cells were transduced with retroviral vectors expressing Mamu-A1*002 and ICP47, a herpes simplex virus 1 protein that inhibits the transporter associated with antigen processing (TAP) (238). Since TAP is necessary for translocation of endogenously-derived peptides from the cytosol into the ER for loading onto nascent MHC class I molecules, MHC class I molecules expressed by 721.221-ICP47 cells are empty and rapidly internalized from the cell surface. Therefore, 721.221-ICP47-A1*002 cells express low levels of surface MHC class I molecules. Because Mamu-A1*002 is not loaded with self-peptide on these cells, the addition of exogenous peptide that binds Mamu-A1*002 ensures that the vast majority of the MHC class I molecules on the cell surface are loaded with this particular peptide. Peptide binding is verified by increased MHC class I surface expression, as Mamu-A1*002 is stabilized once loaded with peptide. This approach allows interrogation of NK cell responses to target cells expressing the same MHC class I molecule but presenting different peptides.

721.221-ICP47-A1*002 cells were pulsed overnight with CD8$^+$ T cell SIV epitopes bound by Mamu-A1*002: $Gag_{71\text{-}79}$ GY9, $Nef_{159\text{-}167}$ YY9, $Env_{788\text{-}795}$ RY8, $Vif_{89\text{-}97}$ IW9, $Env_{760\text{-}768}$ SY9, or $Nef_{169\text{-}177}$ KL9. Peptide-pulsed cells were then stained with CAM and incubated with sorted Mamu-KIR3DL05$^+$ or -KIR3DL05$^-$ NK cells from the same animal that had been expanded on γ-irradiated K562 Clone 9.mbIL21 cells (219). Mamu-KIR3DL05$^-$ NK cells killed all peptide-pulsed cells to a similar extent (Figure 3.1A). In contrast, Mamu-KIR3DL05$^+$ NK

cells were inhibited by target cells pulsed with GY9, YY9, or RY8, but not by target cells pulsed with IW9, SY9, or KL9. This selective inhibition was observed in experiments with cells from four different animals (Figure 3.1B) and was not caused by differences in MHC class I surface expression as all pulsed peptides stabilized Mamu-A1*002 to a similar extent (Figure 3.1C). These results are consistent with our previously published binding data in which Mamu-A1*002 tetramers folded with GY9, YY9, or RY8, but not IW9, bound to Mamu-KIR3DL05*008 (144).

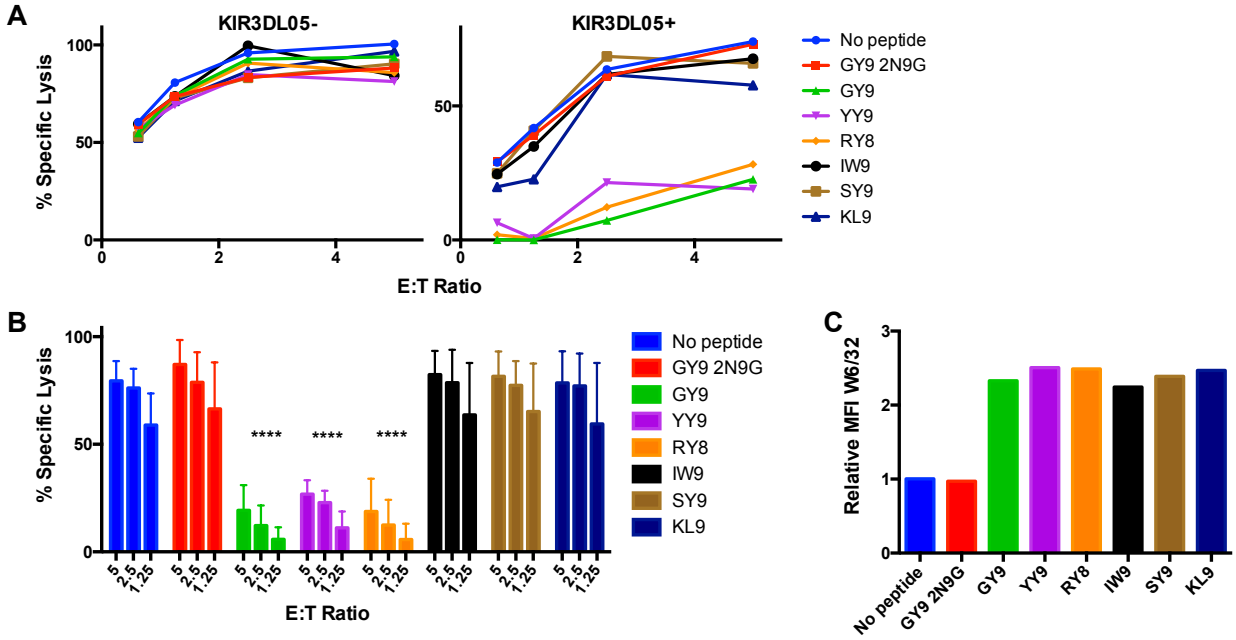

Figure 3.1. Mamu-A1*002 inhibits Mamu-KIR3DL05^{+} NK cells when presenting SIV peptides GY9, YY9, and RY8, but not IW9, SY9, or KL9. (**A**) Mamu-KIR3DL05^{+} and -KIR3DL05^{-} NK cells from the same animal were coincubated with 721.221-ICP47-A1*002 cells pulsed with the SIVmac239 Mamu-A1*002 epitopes indicated. Killing of target cells at the indicated effector:target (E:T) ratios was evaluated by release of CAM from target cells into the culture supernatant. % Specific Lysis is defined as (test release – spontaneous release) / (maximum release – spontaneous release). Results are representative of those obtained with expanded cells from three different animals and the compiled results for Mamu-KIR3DL05^{+} NK cells from these animals at a 5:1 E:T ratio are presented in (**B**). Error bars indicate + 1 SD. Asterisks indicate a significant difference (****$p<0.001$ by ANOVA with Dunnett's test) between coincubation with unpulsed 721.221-ICP47-A*02 cells (No peptide) and those pulsed with the indicated peptide at all E:T ratios shown. (**C**) Stabilization of A1*002 on the 721.221-ICP47-A1*002 cell surface by peptide pulsing was verified by staining with W6/32 antibody and was comparable for all epitopes. GY9 2N9G is a non-A1*002-binding peptide control.

To identify inhibitory and non-inhibitory peptides among the 75 SIV peptides previously shown to bind to Mamu-A1*002 (239), 721.221-ICP47-A1*002 cells pulsed with each of these peptides were incubated with Mamu-KIR3DL05^{+} or -KIR3DL05^{-} NK cells from the same animal in a CAM cytotoxicity assay. All peptides stabilized Mamu-A1*002 surface expression to approximately the same degree (Supplemental Figure 3.1) and Mamu-KIR3DL05^{-} NK cells lysed all pulsed cells to a similar extent (Supplemental Figure 3.2). However, cells pulsed with 28 of the 75 peptides significantly inhibited killing by Mamu-KIR3DL05^{+} NK cells (Figure 3.2). Among these inhibitory peptides are 7 epitopes to which a CD8^{+} T cell response has been previously identified in SIV-infected animals, confirming that these epitopes are presented in vivo. The peptides in Figure 3.2 are presented in order of binding affinity for Mamu-A1*002. Notably, the four highest-affinity Mamu-A1*002-binding SIV peptides are inhibitory and include the immunodominant CD8^{+} T cell eptiopes Nef YY9, Env RY8, and Gag GY9. Due to their high affinity for Mamu-A1*002, these inhibitory peptides may be presented at a higher frequency on the cell surface than other viral peptides, and may therefore be more likely to have an effect upon Mamu-KIR3DL05^{+} NK cell killing of infected cells.

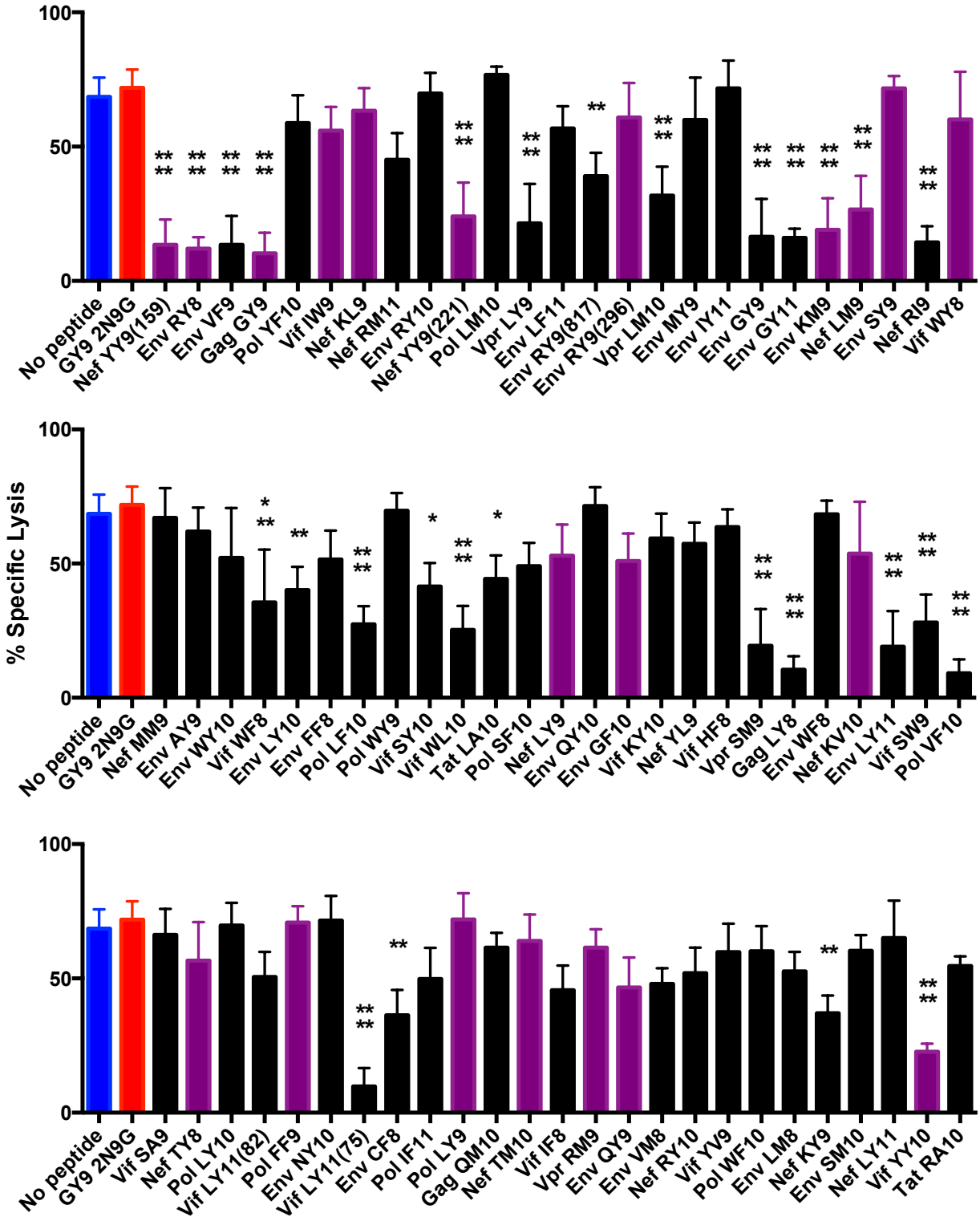

Figure 3.2. Twenty-eight of 75 Mamu-A1*002-binding SIV peptides inhibit Mamu-KIR3DL05^{+} NK cells. Mamu-KIR3DL05^{+} NK cells were coincubated at a 5:1 E:T ratio with 721.221-ICP47-A1*002 cells pulsed with the Mamu-A1*002-binding SIVmac239 peptides indicated. Peptides

Figure 3.2 (Continued). are ordered by affinity of peptide binding to Mamu-A1*002 from highest affinity to lowest and defined CD8$^+$ T cell epitopes are shown in purple. Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. Compiled results are from experiments with expanded cells from three different animals and error bars indicate + 1 SD. Asterisks indicate a significant difference (*p<0.05, **p<0.01, ***p<0.005, ****p<0.001 by ANOVA with Dunnett's test) between coincubation with unpulsed 721.221-ICP47-A1*002 cells (No peptide) and those pulsed with the indicated peptide. Stabilization of Mamu-A1*002 on the 721.221-ICP47-A1*002 cell surface by peptide pulsing was verified by staining with W6/32 antibody and was comparable for all peptides (Supplemental Figure 3.1). GY9 2N9G is a non-A1*002-binding peptide control.

*Changes in C-terminal residues of Mamu-A1*002-presented SIV peptides can alter inhibitory phenotype*

Crystal structures of KIR in complex with ligands have shown that KIRs bind over the C-terminus of MHC class I-presented peptide (145, 150-152). To evaluate the residues responsible for inhibitory and non-inhibitory phenotypes of the SIV peptides described above, peptides with substitutions at C-terminal residues were produced for three inhibitory peptides and one non-inhibitory peptide that bind to Mamu-A1*002 with high affinity: GY9, YY9, RY8, and IW9, respectively. 721.221-ICP47-A1*002 cells pulsed with variant peptides were used as target cells in CAM cytotoxicity assays with Mamu-KIR3DL05$^+$ and -KIR3DL05$^-$ NK cells from the same animal. Mamu-A1*002 stabilization by peptide pulsing was similar for all peptides tested (Supplemental Figure 3.3). Mamu-KIR3DL05$^-$ NK cells killed all targets to a similar extent, but Mamu-KIR3DL05$^+$ NK cells were inhibited to varying degrees by cells pulsed with select variant peptides.

GY9 residue 8 is critically important for facilitating interaction with Mamu-KIR3DL05. A leucine to alanine substitution at this position (GY9 L8A) decreased the inhibitory capacity of the peptide, whereas GY9 with a leucine to tryptophan substitution at the same position (GY9 L8W) was completely non-inhibitory (Figure 3.3A). Variant peptides with a leucine to

phenylalanine or tyrosine substitution at residue 8 (GY9 L8F and GY9 L8Y, respectively) were also non-inhibitory (Supplemental Figure 3.4). The effect of residue 8 substitutions upon Mamu-KIR3DL05^{+} NK cell inhibition by GY9-pulsed cells was reproducible in experiments with NK cells from three different animals (Figure 3.3B). In contrast to the unique effect of variants at GY9 residue 8, YY9 residues 6 through 8 all affect its inhibitory phenotype. Alanine substitution at position 6 (YY9 G6A) resulted in a less inhibitory peptide, whereas an alanine subsitution at position 8 (YY9 R8A) rendered a more inhibitory peptide (Figure 3.3C-D). Decreased inhibition was observed with tryptophan subsitution at YY9 residue 6 or 7 (YY9 G6W, YY9 I7W, respectively) and tryptophan subsitution at position 8 (YY9 R8W) abrogated inhibition of Mamu-KIR3DL05^{+} NK cells in response to YY9-pulsed target cells. Corresponding to its shorter length, RY8 residue 7 is most important for Mamu-KIR3DL05 recognition of Mamu-A1*002-RY8 complexes, as tryptophan substitution at this position (RY8 V7W), and to a lesser extent residue 6 (RY8 R6W), decreases interaction with Mamu-KIR3DL05 (Figure 3.3E-F). Conversely, double substitutions at residue 8 and 9 in the non-inhibitory peptide IW9 (IW9 F8A/W9Y) confer an inhibitory phenotype (Figure 3.3G-H). These results demonstrate that C-terminal residues of all four SIV peptides tested govern Mamu-KIR3DL05 recognition of Mamu-A1*002. The precise characteristics required of an inhibitory peptide are not obvious, but bulky residue substitutions such as tryptophan at the penultimate peptide residue 7 or 8 consistently disrupted the Mamu-A1*002-KIR3DL05 interaction.

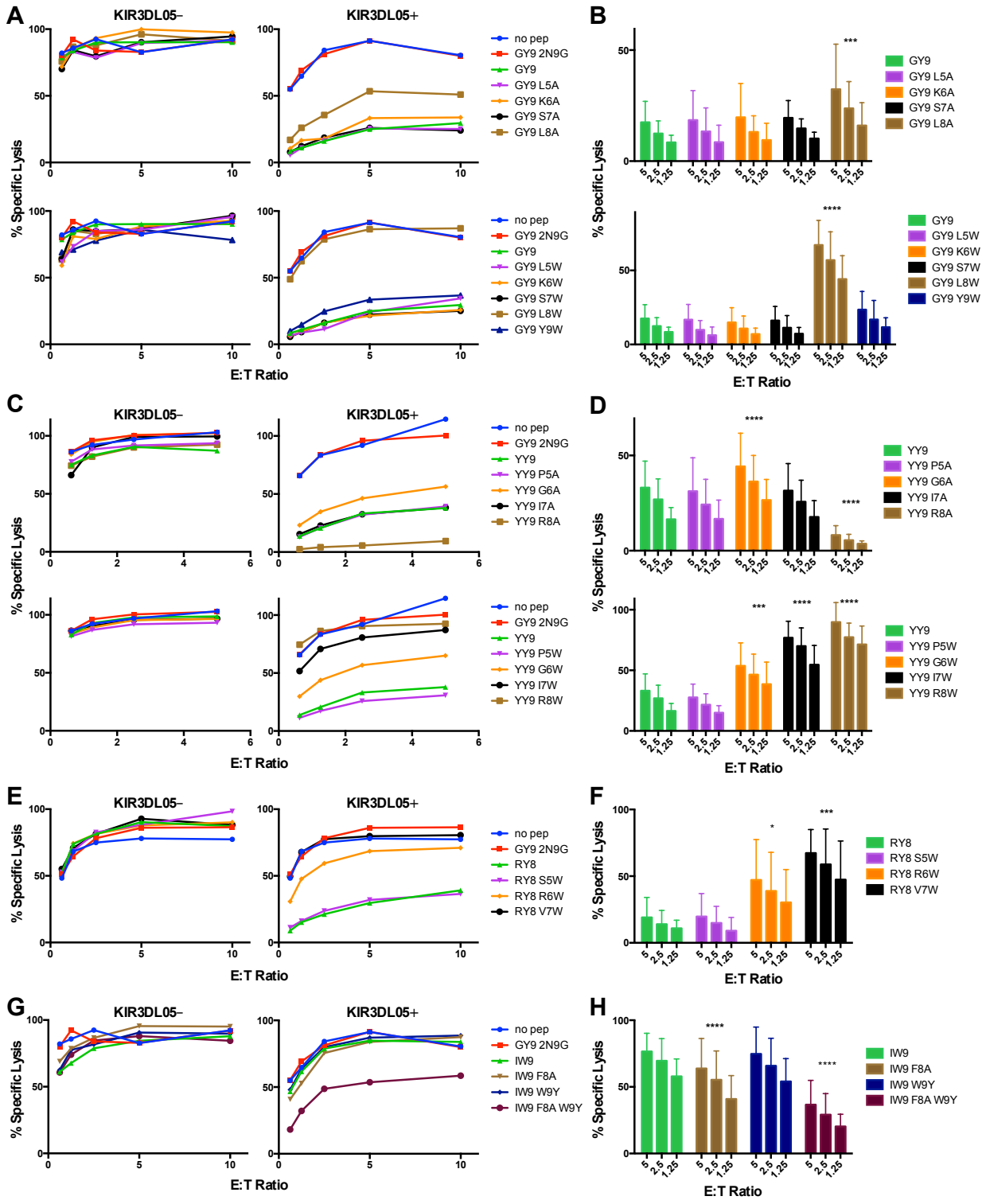

Figure 3.3. Substitutions at peptide residues 6 through 9 can alter inhibitory capacity. Mamu-KIR3DL05^{-} and -KIR3DL05^{+} NK cells from the same animal were coincubated with 721.221-ICP47-A1*002 cells pulsed with variants of GY9 (**A**), YY9 (**C**), RY8 (**E**), and IW9 (**G**) as indicated. Killing of target cells at the indicated E:T ratios was evaluated by release of CAM from target cells into the culture supernatant. Results are representative of those obtained with

Figure 3.3 (Continued). expanded cells from at least two different animals and the compiled results from these animals are presented in (**B**, **D**, **F**, and **H**), respectively. Error bars indicate + 1 SD. Asterisks indicate a significant difference (*p<0.05, ***p<0.005, ****p<0.001 by ANOVA with Dunnett's test) between coincubation with 721.221-ICP47-A1*002 cells pulsed with the wildtype epitope and those pulsed with the indicated epitope variant at all E:T ratios shown. Stabilization of Mamu-A1*002 on the 721.221-ICP47-A1*002 cell surface by peptide pulsing was verified by staining with W6/32 antibody and was comparable for all peptides (Supplemental Figure 3.3). GY9 2N9G is a non-A1*002-binding peptide control.

Remarkably, both GY9 and YY9 have been crystallized in complex with Mamu-A1*002. The crystal structure of Mamu-A1*002 bound to GY9 shows that residue 8 protrudes from the peptide binding cleft (240). In silico substitution at this position to alanine or tryptophan alters the surface over which Mamu-KIR3DL05 is expected to bind (Figure 3.4A). The reduced inhibitory capacity of GY9 L8A may be due to the absence of a necessary contact with the leucine sidechain that protrudes in the wildtype epitope. Conversely, the bulky protrusion of a tryptophan sidechain at this position may sterically hinder interaction with Mamu-KIR3DL05, leading to the non-inhibitory phenotype of the GY9 L8W peptide. Modeling the critical disruptive trypophan substitutions at position 7 or 8 onto the Mamu-A1*002-YY9 crystal structure (241) demonstrates that the substituted sidechain at these two positions is also predicted to protrude out of the peptide-binding cleft (Figure 3.4B), perhaps thereby preventing interaction with Mamu-KIR3DL05. These structures suggest that Mamu-KIR3DL05 recogntion of Mamu-A1*002 is not sensitive to all substitutions in C-terminal peptide residues, but more specifically to substitutions in residues that have a sidechain protruding from the peptide-binding cleft and are therefore accessible for KIR interaction.

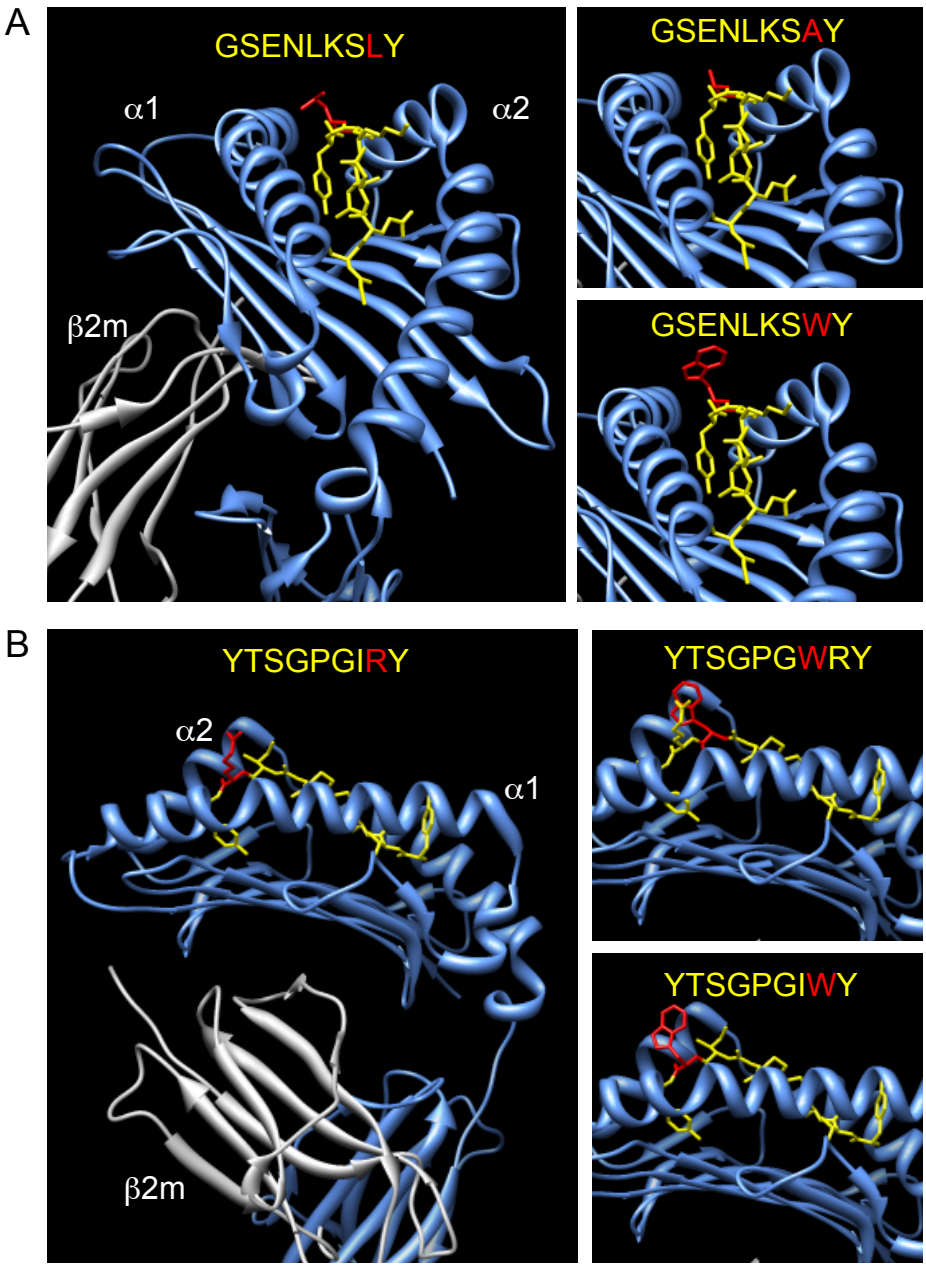

Figure 3.4. Structural implications of peptide modifications of GY9 and YY9. (**A**) Crystal structure of Mamu-A1*002 (blue) loaded with GY9 (yellow) (240). Alanine and tryptophan substitutions at peptide position 8, highlighted in red, are modeled. (**B**) Crystal structure of Mamu-A1*002 (blue) loaded with YY9 (yellow) (241). Tryptophan substitutions at peptide positions 7 and 8, highlighted in red, are modeled.

The inhibitory peptide GY9 dominates over its non-inhibitory variant in peptide mixtures to prevent lysis by Mamu-KIR3DL05^{+} NK cells

To determine whether a viral inhibitory epitope exerts an inhibitory effect when co-presented with non-inhibitory peptide, as would occur on the infected cell surface, peptide mixture experiments were conducted. CAM cytotoxicity assays were performed with 721.221-ICP47-A1*002 cells pulsed with peptide mixtures of GY9 and GY9 L8W. When present at 25% of the total peptide concentration, GY9 exerted nearly its full inhibitory capacity in preventing killing by Mamu-KIR3DL05^{+} NK cells (Figure 3.5A). This result was consistent in experiments with NK cells from three different animals (Figure 3.5B). To investigate whether the inhibitory effect of GY9 could be removed by further dilution, 721.221-ICP47-A1*002 cells were pulsed with a mixture 0.375 μM GY9 L8W and decreasing concentrations of GY9 beginning with the lowest GY9 concentration tested in the previous experiment. Surprisingly, GY9 exerts a detectable inhibitory effect even when diluted to concentrations lower than 10% of the peptide mixture (Figure 3.5C). Similar results were observed with peptide mixtures of RY8 and the non-inhibitory variant RY8 7W (Figure 3.5D-F) The dominance of inhibitory peptides GY9 and RY8 support the hypothesis that viral inhibitory peptides may prevent lysis by KIR-expressing subsets of NK cells when presented on an infected cell surface as part of a peptide mixture.

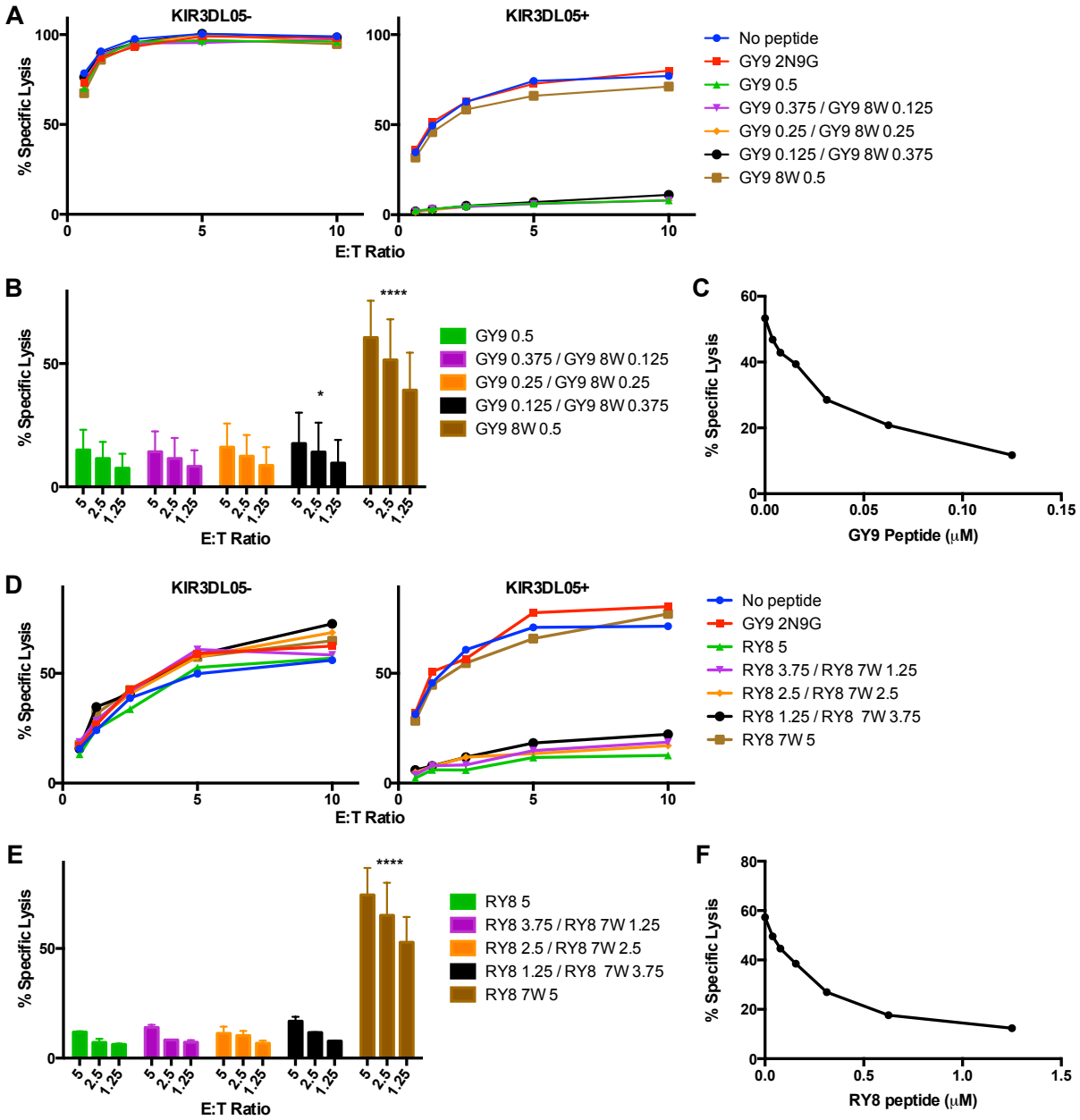

Figure 3.5. Dominance of an inhibitory peptide over the non-inhibitory variant. Mamu-KIR3DL05^{+} NK cells were coincubated with 721.221-ICP47-A1*002 cells pulsed with mixtures of GY9 and GY9 8W (**A**) or RY8 and RY8 7W (**D**) at the μM concentrations indicated. Killing of target cells at the indicated E:T ratios was evaluated by release of CAM from target cells into the culture supernatant. Results are representative of those obtained with expanded cells from at least two different animals and the compiled results from these animals are presented in (**B**) and (**E**), respectively. Error bars indicate + 1 SD. Asterisks indicate a significant difference (*p<0.05, ****p<0.001 by two-way ANOVA with Dunnett's test) between coincubation with 721.221-ICP47-A1*002 cells pulsed with GY9 or RY8, respectively, and those pulsed with the indicated peptide mixture at all E:T ratios shown. Mamu-KIR3DL05^{+} NK cells were coincubated at a 5:1 E:T ratio with 721.221-ICP47-A1*002 cells pulsed with the indicated concentration GY9 in a mixture with 0.375 μM GY9 8W (**C**) or with the indicated concentration of RY8 in a mixture

Figure 3.5 (Continued). with 3.75 μM RY8 7W (**F**). Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. Stabilization of A1*002 on the 721.221-ICP47-A1*002 cell surface by peptide pulsing was verified by staining with W6/32 antibody and was comparable for all peptide mixtures (Supplemental Figure 3.5). GY9 2N9G is a non-A1*002-binding peptide control.

*Mamu-KIR3DL05$^+$ NK cells do not fully suppress SIV replication in Mamu-A1*002$^+$ CD4$^+$ T cells*

Mamu-KIR3DL05 ligand recognition on SIV-infected cells could be affected by Nef-mediated downregulation of Mamu-A1*002 (99). To investigate whether Mamu-KIR3DL05$^+$ NK cells are selectively inhibited in their ability to suppress SIV replication despite Nef-mediated downregulation of Mamu-A1*002, we carried out viral suppression assays. Viral replication was monitored in autologous CD4$^+$ T cells incubated with sorted Mamu-KIR3DL05$^+$ or -KIR3DL05$^-$ NK cells. In assays using cells from Mamu-A1*002$^-$ animals, both NK cell subsets suppressed viral replication to a similar extent (Figure 3.6A). In contrast, viral replication in the presence of Mamu-KIR3DL05$^+$ NK cells was greater than in the presence of Mamu-KIR3DL05$^-$ NK cells for experiments with cells from Mamu-A1*002$^+$ animals. This difference was consistent in experiments performed with cells from three different Mamu-A1*002$^+$ animals (Figure 3.6B). The marked inhibition of Mamu-KIR3DL05$^+$ NK cells, even when Mamu-A1*002 is downregulated by Nef, may be caused in part by the presence of viral peptides that promote Mamu-KIR3DL05 recognition of Mamu-A1*002.

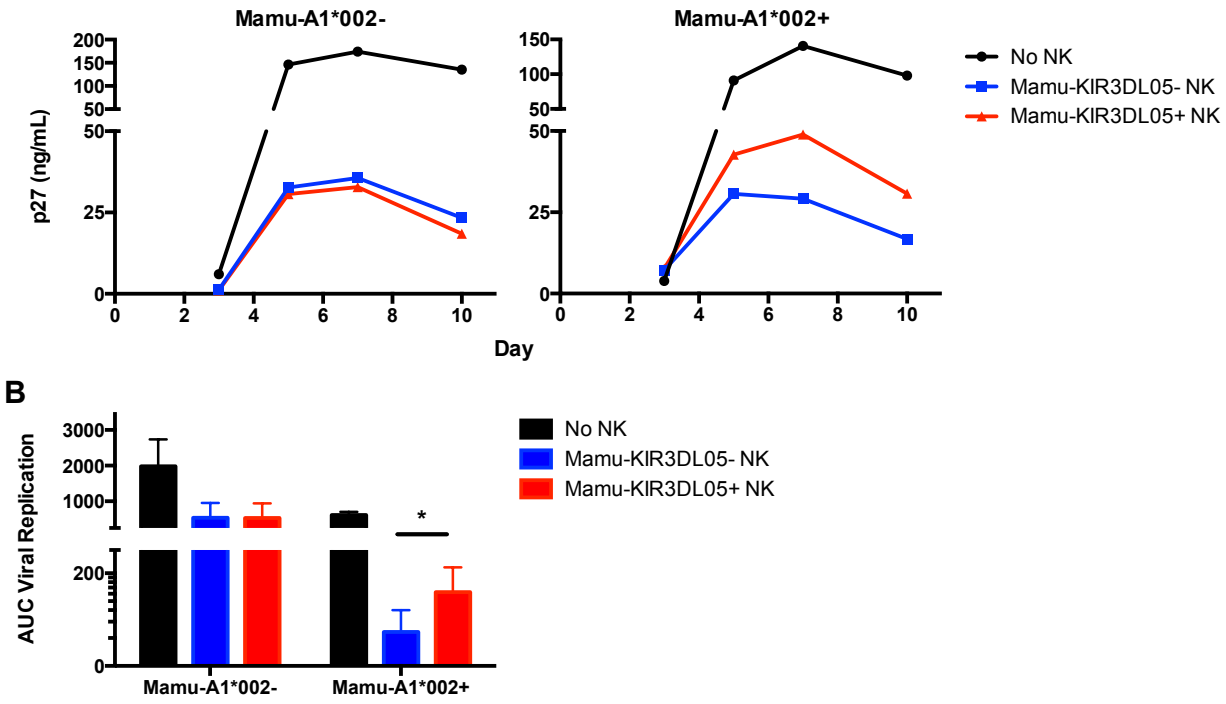

Figure 3.6. Mamu-KIR3DL05^{+} NK cells are inhibited have limited ability to suppress SIV replication in Mamu-A1*002^{+} cells. (**A**) Mamu-KIR3DL05^{+} and -KIR3DL05^{-} subsets of CD3^{-} NKG2A^{+} sorted primary NK cells were incubated with SIV-infected autologous CD4^{+} target cells (2×10^{4} cells) in duplicate wells at a 3:1 E:T ratio. SIV replication in cells from Mamu-A1*002^{-} and -A1*002^{+} animals was determined by measuring the accumulation of SIV p27 in the culture supernatant by ELISA. Each graph is representative of results obtained with cells from three different animals and the compiled results from these animals are presented in (**B**) where AUC is area under the curve. Error bars indicate + 1 SEM, (*$p<0.05$ by paired t-test).

3.6 - Discussion

The importance of KIR-MHC class I interactions in viral infections has been established by genetic studies associating expression of certain KIR and MHC class I molecules with control of viral infections including HIV, HBV, and HCV (176, 177, 194, 196, 242). However, MHC class I-bound peptide can alter KIR ligand recognition, and little is known about the ability of viral peptides to alter inhibition of KIR-expressing NK cell subsets during infection. In this study, we identified 28 SIV Mamu-A1*002-binding peptides, including three immunodominant $CD8^+$ T cell epitopes, that allow ligand recognition by Mamu-KIR3DL05 and inhibit NK cells expressing this KIR. We determined that substitutions at C-terminal positions in these epitopes, such as the introduction of tryptophan at position 7 or 8, can disrupt Mamu-KIR3DL05 ligand recognition. When presented as part of a peptide mixture, an inhibitory peptide was dominant over a non-inhibitory peptide and prevented target cell lysis by Mamu-$KIR3DL05^+$ NK cells. Finally, Mamu-$KIR3DL05^+$ NK cell suppression of viral replication in autologous $CD4^+$ T cells was selectively inhibited in assays with cells from Mamu-$A1*002^+$ animals. These results suggest that viral peptides that alter KIR recognition of MHC class I ligands may affect the activity of KIR-expressing subsets of NK cells, even when presented as a part of a peptide mixture and under conditions of partial Nef-mediated MHC class I downregulation.

This study is the first analysis of differential KIR inhibition mediated by the complete set of viral peptides presented by a single MHC class I molecule. Although only 28 of the 75 peptides tested were inhibitory, the four SIV peptides that bind Mamu-A1*002 with the highest affinity all inhibited Mamu-$KIR3DL05^+$ NK cells very effectively. This enhanced inhibition was not a direct effect of better Mamu-A1*002 binding, as all peptides stabilized Mamu-A1*002 cell surface expression to a similar extent. These four high-affinity peptides may more readily bind

Mamu-A1*002 during MHC class I loading and be presented at a greater frequency on an infected cell surface, thereby increasing the likelihood that they have an effect on Mamu-KIR3DL05^{+} NK cell activity in vivo. Furthermore, among these interactions, the Mamu-A1*002-KIR3DL05 interaction promoted by Gag GY9 is of particularly high affinity. Mamu-A1*002 tetramer folded with this peptide can stain Mamu-KIR3DL05 on primary NK cells (144), whereas other rhesus and human tetramers tested can only stain KIR that are overexpressed. High affinity interactions between Mamu-KIR3DL05 and Mamu-A1*002 presenting peptides such as GY9 may play a role in preventing Mamu-KIR3LD05^{+} NK cell activation in response to SIV-infected cells.

Although Mamu-A1*002 is downregulated by Nef in SIV-infected cells (99), the reduced capacity of Mamu-KIR3DL05^{+} NK cells from Mamu-A1*002^{+} animals to suppress viral replication in autologous T cells demonstrates that despite partial downregulation, there is still sufficient Mamu-A1*002 on the infected cell surface to inhibit Mamu-KIR3DL05^{+} NK cell activity. This observation contrasts with human KIR3DL1^{+} NK cells from HLA-B*57 individuals, which are more active than KIR3DL1^{-} NK cells in response to HIV-infected autologous cells, presumably due to lost HLA-B*57 recognition as a result of Nef expression during infection (183). These KIRs also differ in their effect upon immunodeficiency virus infection: human KIR3DL1 is protective in HIV infection (177) and Mamu-KIR3DL05 is associated with higher viral load in SIV-infected animals (186). The activity of these NK cell subsets suggests that although NK cells expressing some inhibitory KIR, such as KIR3DL1, are activated by infected cells, Mamu-KIR3DL05^{+} NK cells have a reduced capacity to respond to SIV-infected cells, perhaps in part due to Mamu-A1*002 presentation of inhibitory peptides including Gag GY9.

We observed that an inhibitory peptide has a dominant effect in a peptide mixture and prevented activation of Mamu-KIR3DL05^{+} NK cells. In the context of self-tolerance, this dominant effect could prevent errant killing of healthy autologous cells. In general, loss of KIR binding to self-MHC class I molecules can lead to NK cell activation. If non-inhibitory peptide present as a small fraction of total peptide on a healthy cell surface could prevent Mamu-KIR3DL05^{+} NK cell inhibition by Mamu-A1*002, then NK cell killing could result from relatively small changes in the peptide repertoire that may otherwise be harmless. However, a previous study looking at two HLA-Cw*0102-presented peptides, VAP-FA, an inhibitory peptide, and VAP-DA, an antagonistic peptide, found that there was a linear change in activation of KIR2DL2/2DL3^{+} NK cells pulsed with mixtures of the two peptides (157). The VAP-DA antagonistic peptide increased NK cell activity even when present as 10% of the peptide mixture, whereas in this study we only observed an increase in NK cell activity when the non-inhibitory peptide made up 75% or more of the peptide mixture.

The discrepancy between these results may reflect a fundamental difference in the importance of peptide for ligand recognition by KIR2DL and Mamu-KIR3DL molecules. Among the available crystal structures, HLA-Cw3 in complex with KIR2DL2 (150) and HLA-B*57 in complex with KIR3DL01*001 (152), respectively, most closely resemble the KIR-ligand complexes involved in these experiments. Although both structures indicate a KIR contact at position 8 of the MHC class I-bound peptide, KIR2DL2 recognition is more sensitive to amino acid substitutions at this position. The K_D of KIR2DL2 binding to HLA-C is more drastically altered by these substitutions than is the K_D of KIR3DL01*001 binding to HLA-B*57 (150, 152), perhaps indicating a higher peptide-dependence for this interaction and greater sensitivity to antagonism. However, it is unclear whether this structural difference also applies more

broadly to KIR2DL2 recognition of HLA-Cw*0102 and Mamu-KIR3DL05 recognition of Mamu-A1*002. The difference between the inhibitory peptide dominance in these two studies may also be due to the nature of the peptide variants examined. We introduced bulky residues at positions 7 or 8 to potentially disrupt the interaction with Mamu-KIR3DL05, whereas the antagonism caused by the VAP-DA peptide relies on a difference in charge to alter interaction (157). Furthermore, the peptide antagonist VAP-DA induces KIR clustering on the NK cell surface, which normally occurs during NK cell recognition of ligand, but in the case of the antagonistic peptide no signal is transduced from the ITIM (157, 243). This antagonist peptide-MHC class I complex is therefore referred to as a low-affinity binder of KIR3DL2 (243), and may represent a qualitatively different type of disruption than that introduced by bulky residues in our study.

The role of peptide polymorphisms in modulating interaction with KIR and the activity of KIR-expressing NK cell subsets has recently been demonstrated in the context of HIV infection. HIV polymorphisms in Vpu and Env associated with KIR2DL2 have been shown to decrease the capacity of NK cells from KIR2DL2$^+$ individuals to suppress viral replication and increase the binding of a KIR2DL2-Fc fusion construct to infected cells, though the mechanism for this effect has not been defined (188). Conversely, a CD8$^+$ T cell escape variant in a Gag epitope presented by HLA-Cw*0102 decreased KIR2DL2 binding to peptide-pulsed cells (191). HLA-C ligands of KIR2DL2 molecules are not downregulated by Nef. In contrast, the KIR ligand we examine in this paper is downregulated by Nef, and it is therefore surprising that this downregulated MHC class I molecule can still diminish the ability of Mamu-KIR3DL05$^+$ NK cells to suppress viral replication and kill virus-infected cells. The prevalence of inhibitory peptides among the highest affinity Mamu-A1*002-binding SIV peptides may contribute to this observation, and suggest

that perhaps viral mutations in peptides bound by other downregulated MHC class I molecules that promote binding to inhibitory KIR may compensate for Nef-mediated MHC class I downregulation, thereby evading the NK cell immune response.

Previous studies of the effect of MHC class I-bound peptide upon interaction with KIR have been limited to the HLA molecules endogenously expressed by T2 cells, a TAP-deficient cell line used in many peptide-pulsing experiments (157, 184, 191). While it is possible to transduce these cells to express additional MHC class I molecules, the peptide-mediated stabilization cannot be attributed to a specific MHC class I molecule when multiple are expressed. The use of 721.221-ICP47 cells in place of T2 cells provides more flexibility for future studies in looking at peptides bound by any MHC class I molecule. This assay will be of particular use for evaluating whether MHC class I-bound peptides that increase recognition by KIR2DL2 are the mechanism by which KIR2DL2-associated polymorphisms promote KIR2DL2 binding to infected cells, and for investigating whether KIR play a role in the HIV and SIV control associated with certain MHC class I molecules.

In conclusion, we used a novel TAP-inhibited peptide-pulse cytotoxicity assay to investigate the activity of Mamu-KIR3DL05^{+} NK cells against Mamu-A1*002^{+} cells presenting different SIV peptides. Among the inhibitory peptides were immunodominant CD8^{+} T cell epitopes. Peptide mixture assays of inhibitory peptides with non-inhibitory variants that had a tryptophan substitution at residue 7 or 8 demonstrated that the inhibitory peptide was dominant at low concentrations and prevented killing of pulsed cells by Mamu-KIR3DL05^{+} NK cells. Virus suppression by Mamu-KIR3DL05^{+} NK cells was selectively inhibited in cells from Mamu-A1*002^{+} animals despite Nef-mediated downregulation of Mamu-A1*002. Inhibitory viral peptides including GY9, YY9, and RY8 may contribute to the decreased activity of Mamu-

KIR3DL05^{+} NK cells by promoting recognition of Mamu-A1*002 on the surface of SIV-infected cells by this inhibitory KIR.

Chapter 4

Discussion

4.1 - Conclusions

The second chapter of this dissertation defined several ligands for the prevalent rhesus macaque KIR Mamu-KIR3DL01. This study suggests that KIR recognition of MHC-B Bw4 molecules has been maintained since humans and macaques last shared a common ancestor. Moreover, mutational analysis revealed that many of the determinant residues for Mamu-KIR3DL01 ligand recognition are also essential for human KIR3DL1 binding (152), which is surprising because these two KIR are not related and do not have an orthologous relationship. One of the severe limitations of studying rhesus macaque KIR and NK cell populations is that few staining reagents are available to identify KIR-expressing NK cell subsets. This work showed that most allotypes of Mamu-KIR3DL01 can be stained by a pan-human-KIR2D antibody. The Mamu-KIR3DL01 specificity of this antibody doubles the number of rhesus macaque KIR that can be identified by antibody staining. Together with the definition of ligands for this KIR, this study will be foundational for further studies of the frequency of KIR-expressing NK cell populations in vivo.

The third chapter of this dissertation demonstrated that approximately one-third of SIV peptides that bind Mamu-A1*002 promote interaction with Mamu-KIR3DL05 and inhibit Mamu-KIR3DL05^{+} NK cells. A novel cytotoxicity assay approach was used to evaluate these peptides by monitoring NK cell killing of peptide-pulsed cells transduced with ICP47, which inhibits TAP, and Mamu-A1*002. This assay allows interrogation of NK cell activity in response to cells that express the same MHC class I molecule, Mamu-A1*002, but present different peptides. Inhibitory peptides were dominant and prevented killing by Mamu-KIR3DL05^{+} NK cells when presented in a mixture with non-inhibitory variants that had a tryptophan substitution at residue 7 or 8. Furthermore, despite Nef-mediated downregulation of Mamu-A1*002, viral

suppression by Mamu-KIR3DL05^{+} NK cells was selectively inhibited with cells from Mamu-A1*002^{+} animals. These results suggest that inhibitory viral peptides that promote Mamu-KIR3DL05 binding to Mamu-A1*002 may diminish the activity of Mamu-KIR3DL05^{+} NK cells, even when these peptides are presented as a part of a peptide mixture and under conditions of partial Nef-mediated Mamu-A1*002 downregulation.

4.2 - Coevolution of KIR and MHC class I

Examination of KIR and MHC class I molecules in primate species illustrates the coevolution of these receptors and ligands. This relationship is evident in the differences in KIR and MHC class I repertoires between macaques and humans. Corresponding to the lack of an HLA-C orthologue in rhesus macaques (207), these animals also lack the KIR2D molecules that recognize MHC-C molecules (211). More strikingly, the emergence of an HLA-C orthologue in orangutans, Popy-C, coincides with the expression of KIR2D molecules (201). Notably, these KIR2D expressed by orangutans all recognize C1 MHC-C molecules that have an asparagine at position 80, corresponding to the epitope present on Popy-C molecules, whereas human KIR2D molecules can recognize MHC-C with either a C1 or a C2 epitope. However, there is also an orangutan KIR that has dual specificity for both C1 and C2 Popy-C molecules (244). The presence of this KIR in the absence of a Popy-C C2 ligand suggests that KIR may provide additional selective pressure for the development of MHC class I ligands that they recognize.

Although KIR and MHC class I are coevolving, there may also be functional advantages to having MHC class I molecules that are not recognized by KIR. The second chapter of this dissertation demonstrated that KIR recognition of Bw4 ligands occurs in both humans and rhesus macaques by a largely similar pattern of recognition (245). However, HLA-B molecules with the

alternate Bw6 motif are not recognized by KIR in humans. In contrast, Mamu-KIR3DL05 recognizes two Bw6-like ligands in rhesus macaques, Mamu-A1*002 and -A3*1303 (144). A potential advantage for humans in having many MHC class I molecules, including Bw6 molecules, that are not KIR ligands could arise from allowing greater variation between individuals in the number of liganded KIRs expressed. If, for example, an individual expressed two Bw6 molecules, any KIR3D molecules that recognize HLA-B Bw4 molecules will not have ligands. Although expressing some KIR in conjunction with their MHC class I ligands is protective in viral infection, these interactions may be disadvantageous for other NK cell functions. The balancing act that exists in KIR immunogenetics is demonstrated by the maintenance of two types of KIR haplotypes in humans: haplotype A, which is advantageous in infection, and haplotype B, containing more activating KIR, which is advantageous in reproduction (246). Under such complicated selection, coevolution of KIR and MHC class I molecules may dictate that some MHC class I remain unrecognized by KIR.

4.3 - Specificity and NK cells

SIV peptide modulation of Mamu-A1*002 recognition by Mamu-KIR3DL05 contributes to a growing body of literature suggesting that NK cell responses are not as innate and non-specific as originally thought. Canonical innate immune responses are not antigen-specific, but instead provide non-specific protection from pathogens. In contrast, the peptide preference in inhibitory KIR ligand recognition has been well-documented (142, 146, 156, 157, 160, 233-236) and an antagonistic peptide may activate NK cells by altering ligand recognition by an inhibitory KIR recognition (157). Instead of a non-specific response to the absence of MHC class I expression as “missing self” (104), this peptide-dependent method of NK cell activation suggests

the recognition of "altered self" wherein a change in the peptides presented by an MHC class I molecule can affect KIR recognition and NK cell activity. The association of HIV polymorphisms with the expression of certain KIRs suggests that these peptide-selective KIR-MHC class I interactions are important during viral infection, although the mechanism of selection for these viral polymorphisms has not yet been demonstrated (188). Furthermore, NK cells can respond to HIV peptides (247), perhaps due to a mechanism involving recognition of presented viral peptides as altered self. In NK cell development KIR are expressed stochastically until an NK cell is sufficiently inhibited by self-MHC class I (132), which presumably includes the normal repertoire of self-peptides presented by that MHC class I molecule. Thus, peptide-mediated NK cell activation may be specific to non-self or unhealthy cell states in which the regular peptide presentation is altered and affects KIR recognition of ligands. Although KIR do not have diversity analogous to that provided by genetic rearrangement of the T cell receptor, the high degree of KIR polymorphism combined with the effect of MHC class I-bound peptide upon KIR recognition create an antigen-specificity of another sort.

Another facet of the specificity of NK cells is the demonstrated ability of these cells to develop antigen-specific memory responses in some cases. This phenomenon has occurred with $Ly49H^+$ NK cells that recognize MCMV-infected cells by m157 expression and can generate a secondary immune response (248) and with liver NK cells that mediate a contact hypersensitivity response to haptens (249) and antigens from influenza, vasicular stomatitis virus, and HIV (250). These studies indicate that NK cells can develop memory to a broad range of antigens, but the receptors responsible for the viral and hapten memory have not been identified. Notably, the expression of Ly49 receptors is not altered in these memory cells, and memory populations can be developed after sensitization in multiple mouse strains that have different Ly49 and MHC

class I backgrounds (250). These characteristics suggest that MHC class I recognition by NK cell receptors is not the mechanism for memory development in this system. These memory-generating experiments have all been performed in mice and it has not yet been determined if primate NK cells can produce similar memory responses. However, it is important to note that many of these murine memory NK cell responses are specific to liver NK cells that express the homing marker CXCR6 (250). It would be difficult to isolate similar tissue-resident NK cell populations from humans for experiments, and investigation of tissue-resident memory NK cell responses in higher animals may therefore be most effective in a non-human primate models like the rhesus macaque.

4.4 - Future Directions

Because so few rhesus macaque KIR ligands have been defined, future studies must continue to determine which KIR and MHC class I molecules interact. The system used in this dissertation to detect ligand for Mamu-KIR3DL01 is quite resource-intense, involving sorting and lengthy culture of primary cells. An advantage of working with primary NK cells is that they express endogenous levels of KIRs. In contrast, stable cell lines would over-express KIR and may therefore be inhibited by weak-binding ligands that would not inhibit KIR on primary NK cells. However, as a general method of screening for KIR ligands, the use of primary NK cells is terribly restricted by the availability of specific staining reagents for rhesus macaque KIRs. To date, only Mamu-KIR3DL05 and -KIR3DL01, the KIRs examined in this study and for which ligands have been identified, can be specifically stained with tetramer or antibody (144, 230, 245). Other mouse monoclonal antibodies have been generated against rhesus macaque KIR, but the utility of these antibodies is limited because they cross-react with with multiple KIRs (230).

As an alternative to primary NK cells, in light of the limitations, a more scalable approach for KIR ligand identification could evaluate the cytotoxicity of NK cell lines transduced with distinct KIRs against a panel of MHC class I-expressing cell lines. However, our attempts to produce these NK cell lines have so far been unsuccessful. Although transduction of NKL and NK92 cell lines with Mamu-KIR3DL01 or -KIR3DL05 resulted in KIR surface expression, these NK cell lines were not inhibited by the ligands of these KIRs (data not shown). A comparison between human KIR3DL1 and Mamu-KIR3DL01 transmembrane and cytoplasmic domains, which would be responsible for interaction with downstream effectors of ITIM signaling, reveals that there is approximately 25% amino acid variation between these KIR (Figure 4.1). These variations may cause an incompatibility between rhesus macaque KIR signaling and human cell lines. Ongoing work to produce chimeric KIR with extracellular domains of rhesus macaque KIRs and transmembrane and cytoplasmic domains of human KIR will attempt to overcome this obstacle and define ligands for rhesus macaque KIRs for which specific staining reagents are not available.

Transmembrane Domain

```
                      350
Mamu-KIR3DL01*001 VLIGTSVAMILFT-IFFFLL
KIR3DL1*001       I......VI...ILLL....
```

Cytoplasmic Domain

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                  360       370       380       390       400       410       420       430       440
Mamu-KIR3DL01*001 HRWCSNKKNAAVMDQEPAGDRTVNREDPDEQDPQEVTYAQLDHRVLTQGKITCPSQRPRRPPTDTSVYTELPNAEPRSKVVFYP
KIR3DL1*001       .L.................N..A.S..S.....E.........C.F..R...R.....KT.....IL.......K......SC.
```

Figure 4.1. Amino acid alignment of transmembrane and cytoplasmic domains of Mamu-KIR3DL01*001 and human KIR3DL1*001.

In addition to general screening for rhesus macaque KIR ligands, the Mamu-KIR3DL01 ligands that we identified suggest a few more specific avenues for research. In particular, Mamu-B*017 is a MHC class I molecule of particular interest due to its association with control of SIV infection (72) and is closely related to the Mamu-B ligands for Mamu-KIR3DL01. Because the

Mamu-KIR3DL01 allotypes in this study recognized similar MHC class I molecules, Mamu-B*17 may be recognized by one of the 23 other allotypes of Mamu-KIR3DL01 (223) or another related KIR. Alternatively, binding of Mamu-B*017 to KIR may be affected by SIV peptides, and investigation of potential Mamu-KIR3DL01 interaction with Mamu-B*017 presenting the 50 SIV peptides that bind to Mamu-B*017 (251) would be another exciting avenue of research. Identification of the KIRs that recognize this protective MHC class I allotype will be very important in determining if Mamu-B*017-KIR interactions play a role in the viral control with which it is associated.

Analysis of viral evolution in MHC class I-defined animals that do or do not express Mamu-KIR3DL05 would be an interesting followup to our finding that certain Mamu-A1*002-bound SIV peptides promote interaction with Mamu-KIR3DL05. Selective accumulation of viral variants in Mamu-A1*002^{+}Mamu-KIR3DL05^{+} animals would warrant investigation by the peptide-pulse cytotoxicity assay to evaluate whether these changes were selected for by increasing inhibition of Mamu-KIR3DL05^{+} NK cells. Variants could accomplish this increased inhibition in two ways: by increasing the inhibitory capacity of an SIV peptide that binds to Mamu-A1*002 (239), or by generating a new inhibitory Mamu-A1*002-bound peptide by mutation in the anchor residues that are bound by this MHC class I molecule. Because this anchor residue pattern is well-documented for Mamu-A1*002, in silico prediction of binding to this MHC class I molecule may be useful to detect accumulation of such changes. These predictions could then be validated by stabilization of surface Mamu-A1*002 on 721.221-ICP47-A1*002 cells as a measure of Mamu-A1*002 binding. If these inhibitory peptides are involved in the viral pathogenesis that leads to higher viral load in Mamu-KIR3DL05^{+} animals (186), one would expect that polymorphisms that increase the inhibitory capacity of Mamu-A1*002-bound

peptides would accumulate selectively in Mamu-A1*002^{+}Mamu-KIR3DL05^{+} animals to inhibit Mamu-KIR3DL05^{+} NK cells and promote viral replication.

Unfortunately it was not possible to investigate the in vivo relevance of the most important inhibitory epitopes identified in this dissertation, Gag GY9 and Nef YY9. Generating full-length SIVmac239 with a mutation at position 8 of GY9 to any of the non-inhibitory variants identified (L8F, L8W, and L8Y substitutions) produces non-replicating virus (data not shown). Furthermore, mutation at position 7 and 8 of YY9 interferes with Nef-mediated downregulation of MHC class I (252). Therefore, the introduction of changes in YY9 intended to alter the inhibitory epitope to be non-inhibitory (I7W and R7W substitutions) into full-length SIVmac239 would result in higher expression of the Mamu-KIR3DL05 ligand Mamu-A1*002 on the infected cell surface, confounding results. Nef is also responsible for downregulating other NK cell receptor ligands (122, 253) and substitutions at these residues of YY9 may therefore have further unintended effects upon NK cell activity in response to SIV-infected cells.

Our findings, together with other recently published work identifying ligands and staining reagents for rhesus macaque KIRs, make it possible to follow KIR-expressing NK cell populations longitudinally in animals that do or do not express the ligand for a given KIR. This prospect is of particular interest during viral infection to monitor how KIR-expressing populations change, in terms of function, localization, and frequency, during the course of infection. Monitoring KIR-expressing NK cell populations in the gut mucosa during SIV infection may better characterize the role of KIR-expressing NK cell subsets in acute and chronic immunodeficiency virus infection, as it is obviously not possible to extensively sample gut NK cell populations in HIV patients.

The data presented in this dissertation demonstrate that rhesus macaque Mamu-KIR3DL01 and human KIR3DL1 recognition of Bw4 ligands exhibit a distinct but overlapping pattern of recognition and suggests that Bw4 recognition has been maintained since the divergence of apes and Old World monkeys. Furthermore, I have demonstrated that the peptide preference of Mamu-KIR3DL05 recognition causes selective inhibition of Mamu-KIR3LD05^{+} NK cells in response to Mamu-A1*002^{+} cells presenting inhibitory SIV peptides. Due to the dominant effect of these inhibitory viral peptides, they may inhibit Mamu-KIR3DL05^{+} NK cells even when presented on the infected cell surface as part of a mixture of self and viral peptides and under conditions of partial MHC class I downregulation. The results of these studies, and the novel use of a KIR-specific antibody and ICP47-transduced peptide-pulse cytotoxicity assay, provide a foundation for further investigations of KIR-MHC class I interactions in SIV infection. The investigation of liver-resident and gut-resident NK cells that are not available for study in HIV patients will be essential to better understanding of the role of KIR-expressing NK cells in immunodeficiency virus infections.

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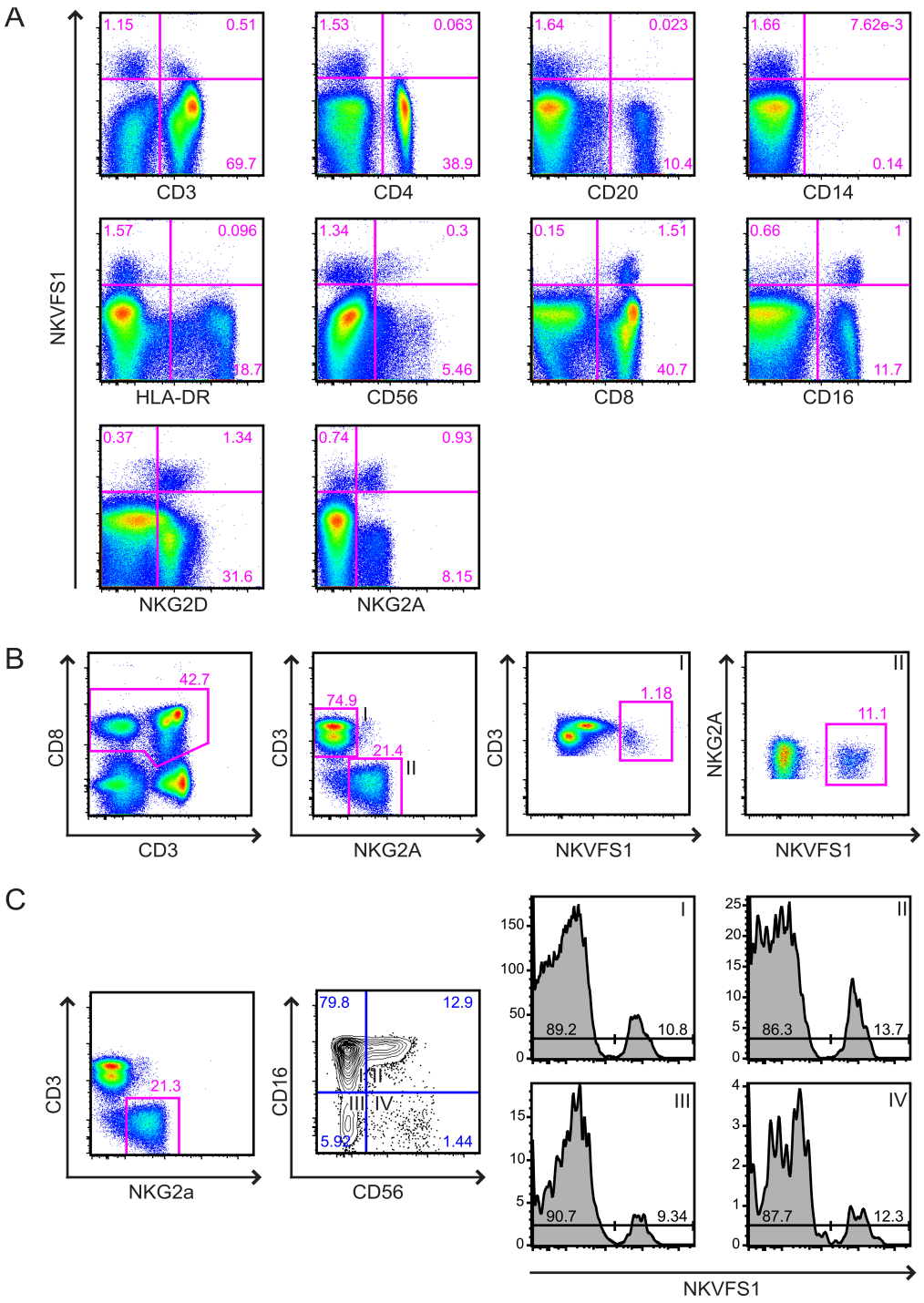
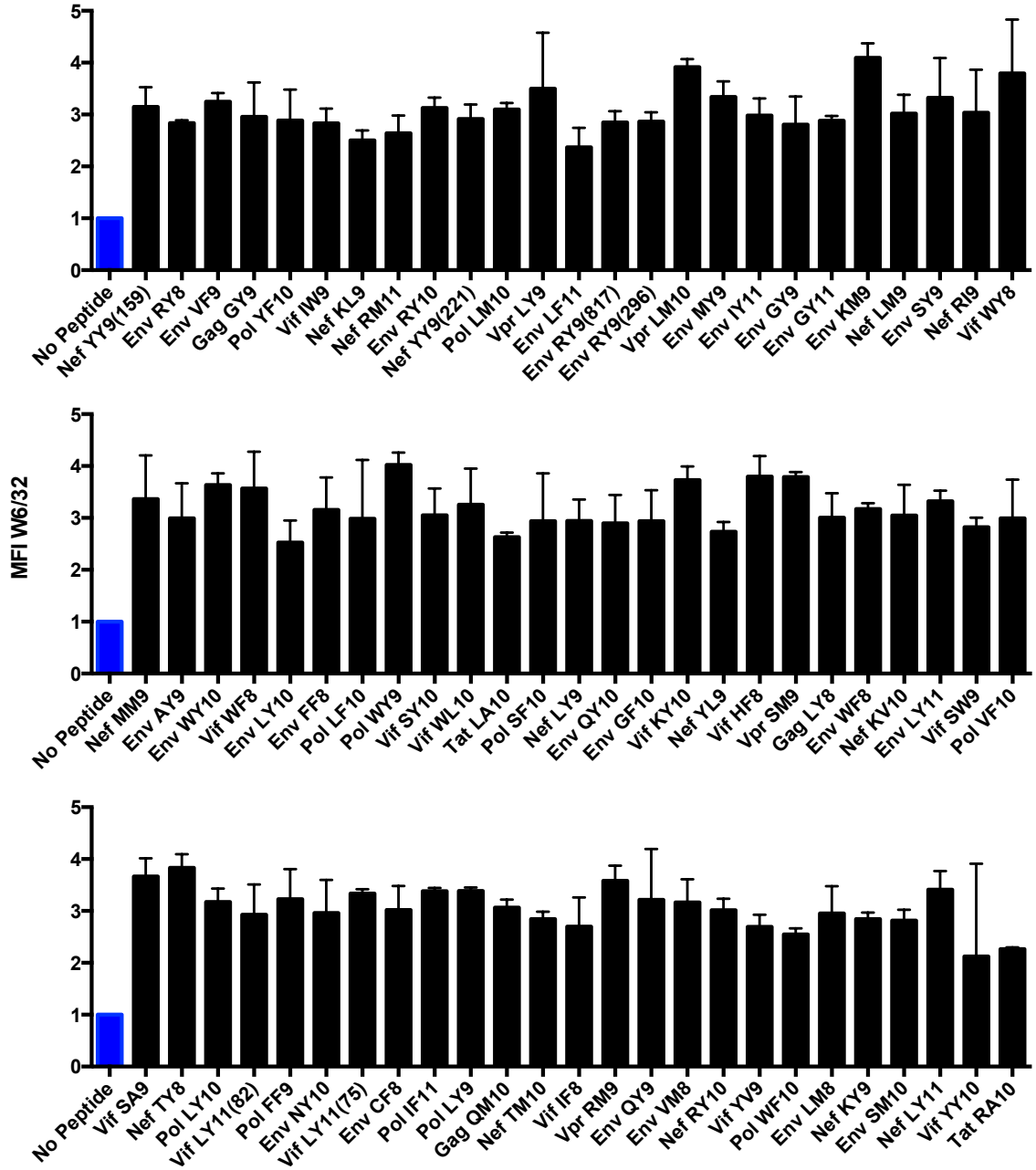
Appendix A

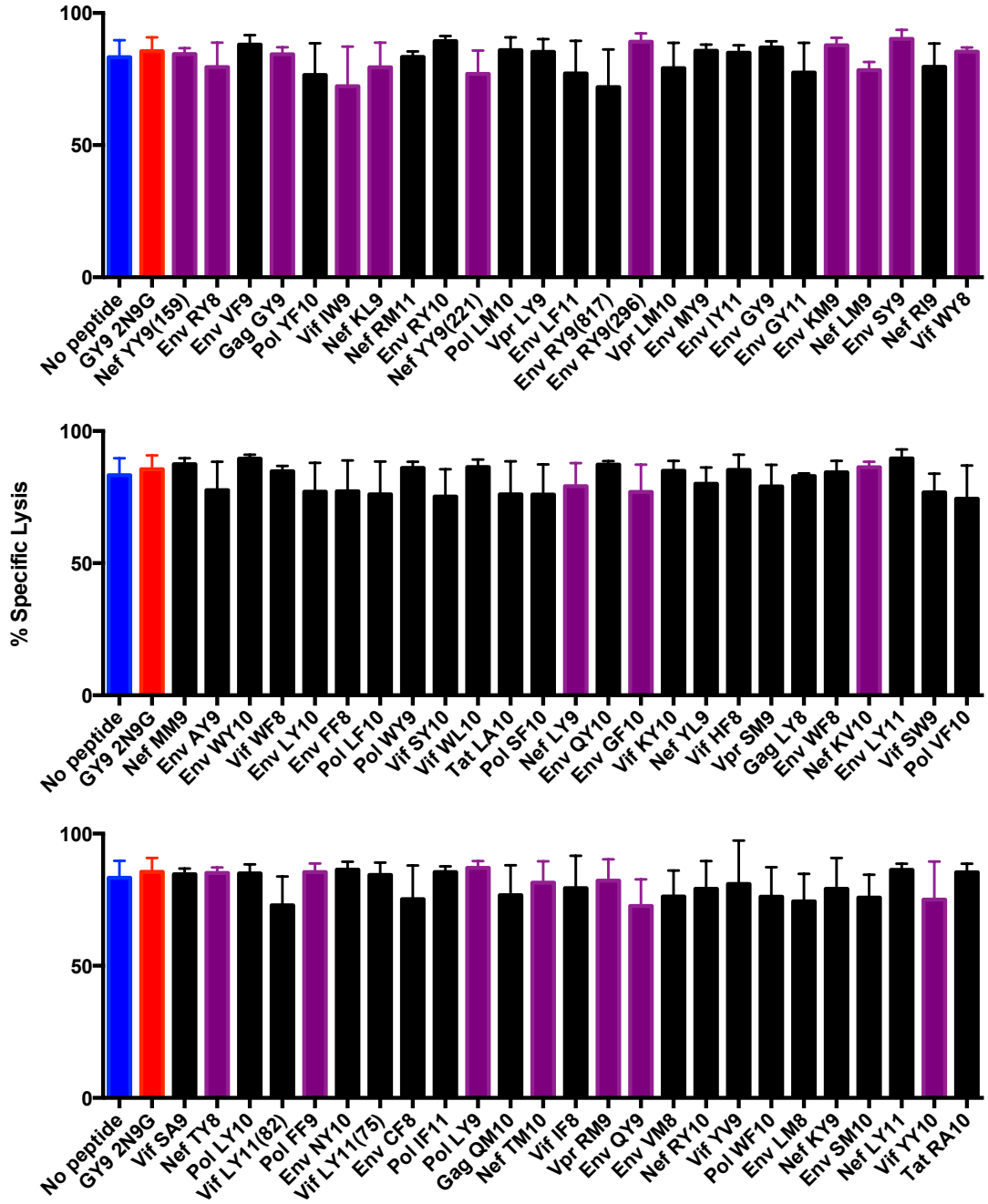
Supplemental Figures



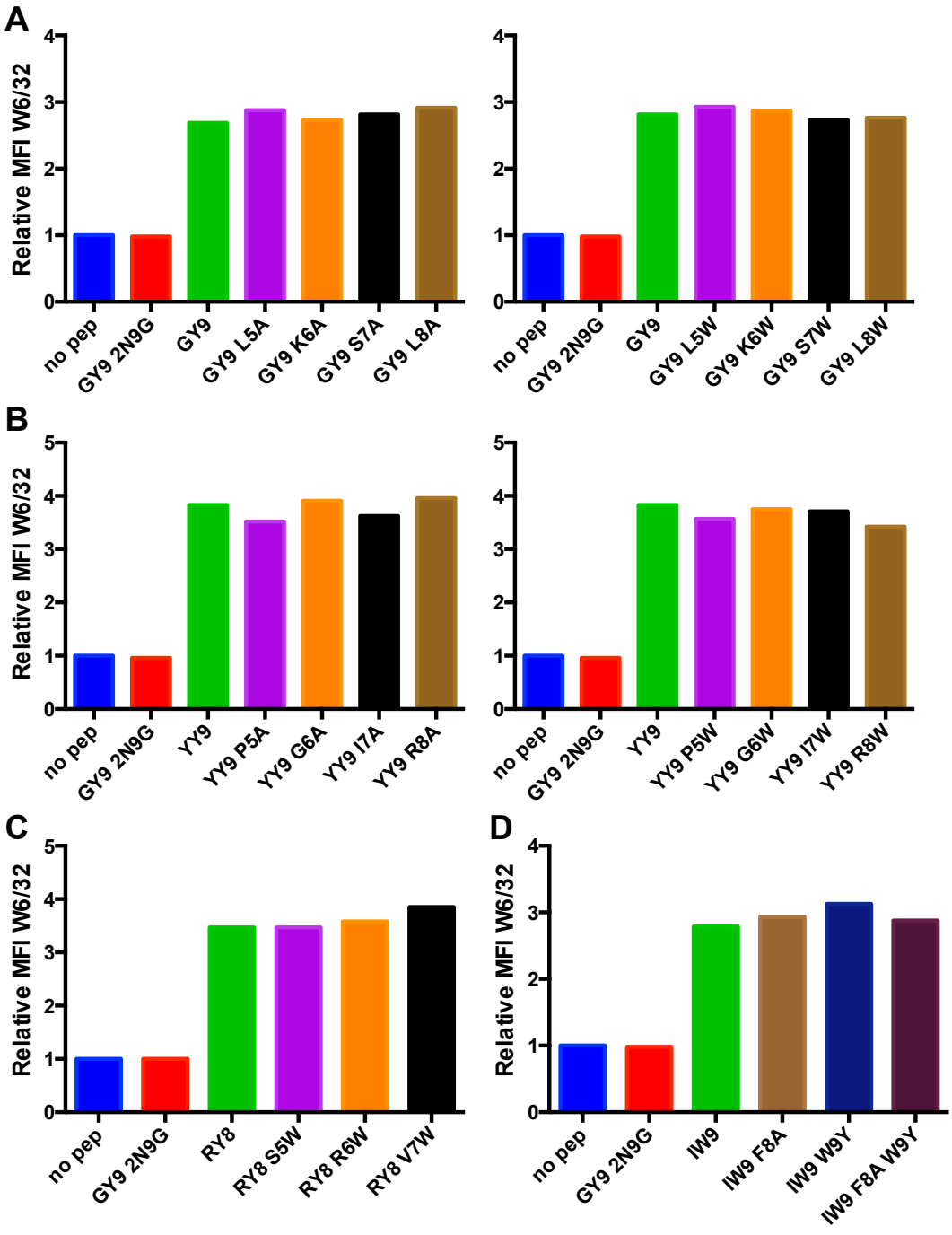
Supplemental Figure 2.1. Pan-KIR2D Ab NKVFS1 stains NK cells in some rhesus macaques. (**A**) NKVFS1 staining of singlet lymphocytes is shown versus staining for different cell-type markers. (**B**) NKVFS1 staining of $CD8^{+}CD3^{+}$ and $CD8^{+}NKG2A^{+}$ cells. (**C**) NKVFS1 staining in the four $CD3^{-}NKG2A^{+}$ NK cell populations defined by CD16 and CD56 expression. Results representative of staining of PBMC from 3 animals.



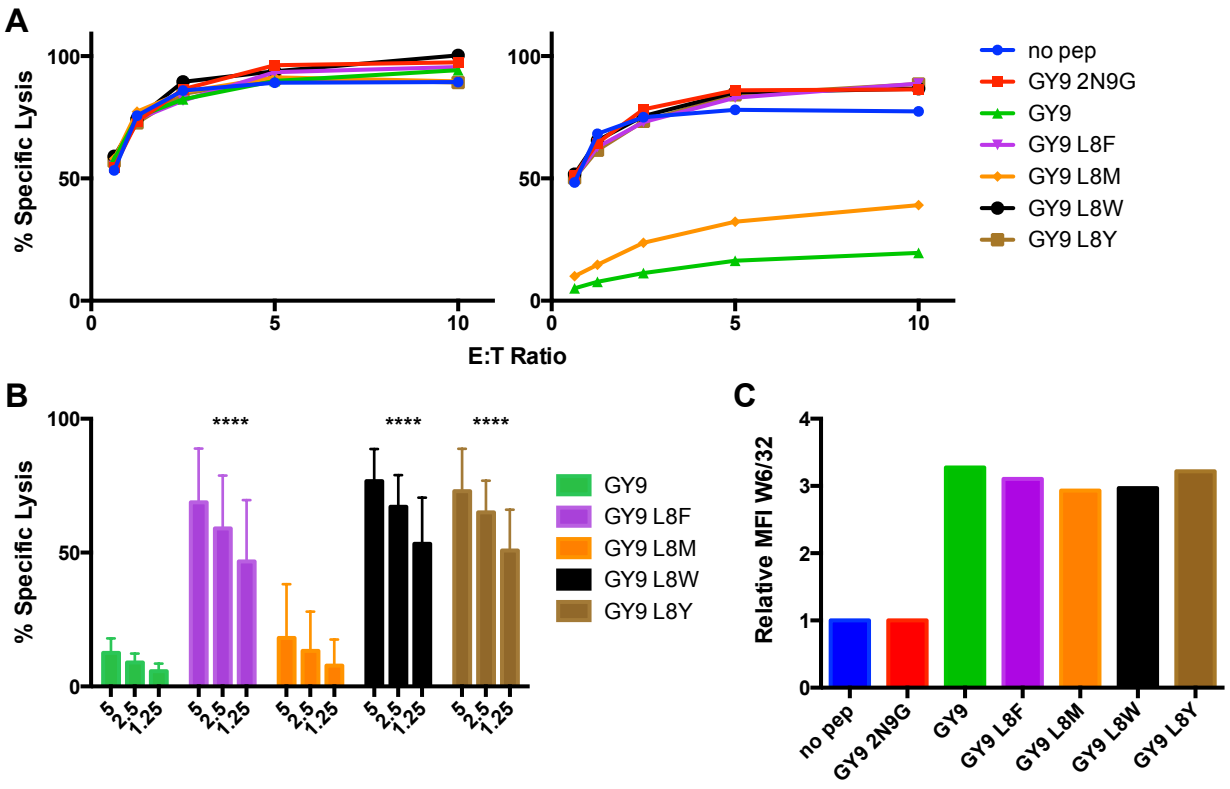
Supplemental Figure 3.1. Stabilization of cell surface Mamu-A1*002 expression by peptide pulse. 721.221-ICP47-A1*002 cells pulsed with the Mamu-A1*002-binding SIVmac239 peptides indicated were stained with pan-MHC class I antibody clone W6/32 to evaluate Mamu-A1*002 surface stabilization. Data summarized from two independent experiments and error bars indicate +1 SD.



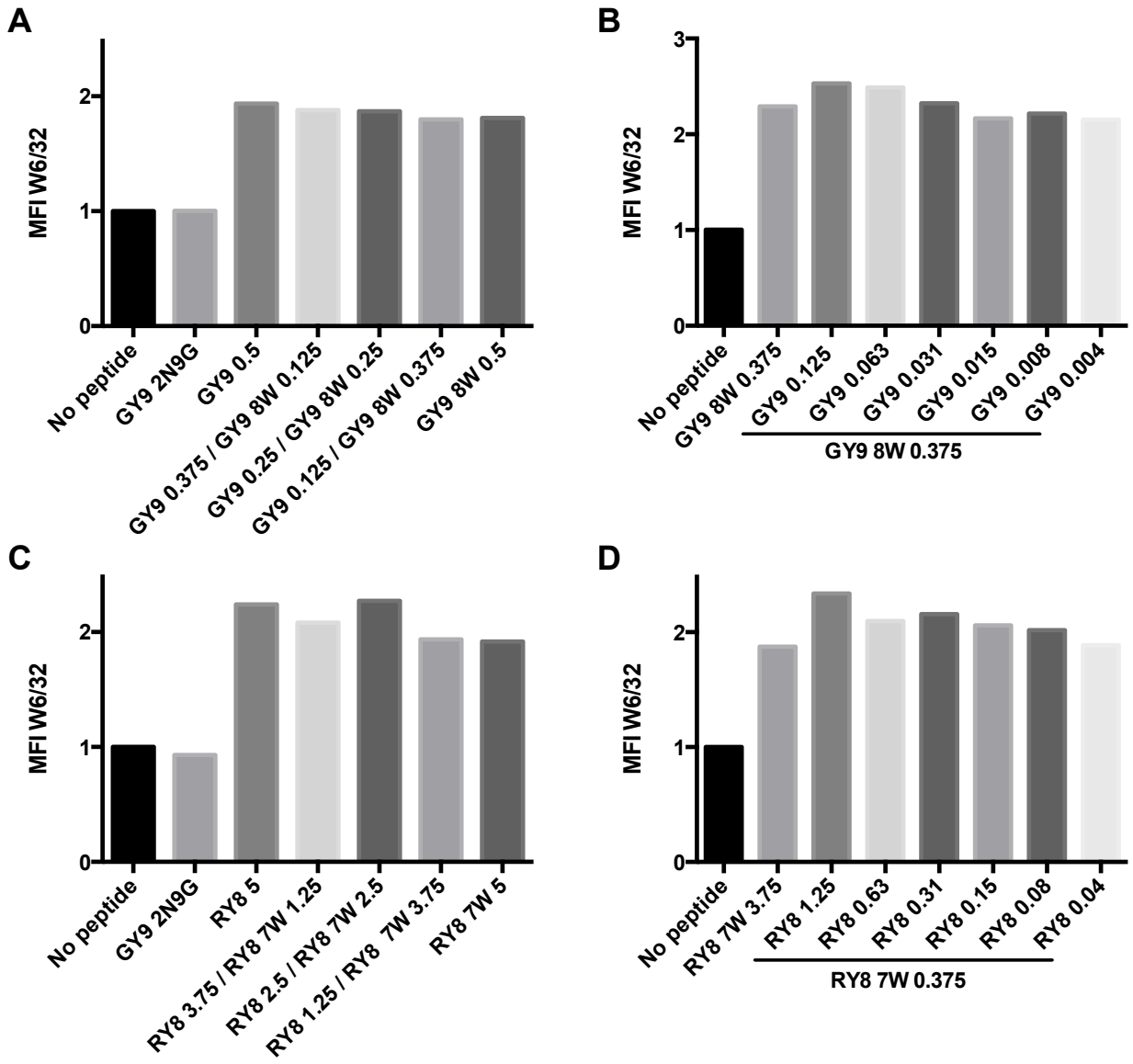
Supplemental Figure 3.2. Lysis of peptide-pulsed cells by Mamu-KIR3DL05^{-} NK cells. Mamu-KIR3DL05^{-} NK cells were coincubated at a 5:1 E:T ratio with 721.221-ICP47-A1*002 cells pulsed with the Mamu-A1*002-binding SIVmac239 peptides indicated. Peptides are ordered by affinity of peptide binding to Mamu-A1*002 from highest affinity to lowest and defined CD8^{+} T cell epitopes are shown in purple. Killing of target cells was evaluated by release of CAM from target cells into the culture supernatant. Compiled results are from experiments with expanded cells from three different animals and error bars indicate + 1 SD. GY9 2N9G is a non-A1*002-binding peptide control.



Supplemental Figure 3.3. Stabilization of cell surface Mamu-A1*002 by peptide pulse. 721.221-ICP47-A1*002 cells pulsed with the Mamu-A1*002-binding SIVmac239 peptide variants indicated were stained with pan-MHC class I antibody clone W6/32 to evaluate Mamu-A1*002 surface stabilization. Data representative of at least two independent experiments.



Supplemental Figure 3.4. Abrogation of GY9 inhibitory capacity by aromatic amino acid substitutions at residue 8. (**A**) Mamu-KIR3DL05^{-} and -KIR3DL05^{+} NK cells were coincubated with 721.221-ICP47-A1*002 cells pulsed with variants of GY9. Killing of target cells at the indicated E:T ratios was evaluated by release of CAM from target cells into the culture supernatant. Results are representative of those obtained with expanded cells from two different animals and the compiled results from these animals are presented in (**B**). Error bars indicate + 1 SD. Asterisks indicate a significant difference (****$p<0.001$ by ANOVA with Dunnett's test) between coincubation with 721.221-ICP47-A1*002 cells pulsed with GY9 and those pulsed with the indicated GY9 variant at all E:T ratios shown. (**C**) Stabilization of Mamu-A1*002 on the 721.221-ICP47-A1*002 cell surface by peptide pulsing was verified by staining with W6/32 antibody. GY9 2N9G is a non-A1*002-binding peptide control.



Supplemental Figure 3.5. Stabilization of cell surface Mamu-A1*002 by peptide mixtures. 721.221-ICP47-A1*002 cells pulsed with mixtures of two peptides at the μM concentrations indicated were stained with pan-MHC class I antibody clone W6/32 to evaluate Mamu-A1*002 surface stabilization. Data representative of at least two independent experiments.